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Synthesis and pharmacological evaluation of thienocinnolin-3-(2*H*)-ones, bioisosters of antihypertensive and antithrombotic benzo(*h*)cinnolinones

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Summary — A number of thienocinnolin-3-(2*H*)-ones (**2b**, **c**; **3a**, **b**) have been synthesized and tested for their pharmacological profile. These were compared with the bioisoster 8-acetylamino-4,4*a*,5,6-tetrahydrobenzo(*h*)cinnolin-3-(2*H*)-one **1**, which we reported to be a potent antihypertensive and antithrombotic agent. Binding studies on phosphodiesterase (PDE) isoenzymes indicate that the test compounds exhibited a modest affinity towards PDE III (**2c**, **3a**, **b**) and PDE V (**2b**, **c**). *In vivo* tests indicated that only **3b** displayed antihypertensive properties comparable to the model while all the new derivatives exhibited lower hypotensive activity. All compounds, with the exception of **2b**, were more potent than **1** in inhibiting collagen-induced platelet aggregation. Molecular mechanics calculations were performed on compounds **2** and **3** which were compared with the model **1**.

tetrahydrobenzo(*h*)cinnolin-3-(2*H*)-ones / tetrahydrothienocinnolin-3-(2*H*)-ones / PDE isoenzymes / antihypertensive activity / hypotensive activity / antiplatelet aggregation activity

Introduction

In the course of our studies on antihypertensive and antithrombotic tricyclic pyridazinones acting, or at least partly, through phosphodiesterase (PDE) III inhibition, 8-acetylamino-4,4*a*,5,6-tetrahydrobenzo(*h*)cinnolin-3-(2*H*)-one **1** proved to be the most potent as both an antihypertensive agent (E_{50} = 3.1 mg/kg po, rat) and an antithrombotic agent (100% protection at an equimolar dose to 20 mg/kg acetylsalicylic acid, mouse) [1].

Unexpectedly, 8-acetylamino-4,4*a*,5,6-tetrahydrothieno(2,3-*h*)cinnolin-3-(2*H*)-one **2a**, a bioisoster of **1**, was found to be deprived of significant antihypertensive properties. A possible explanation for its inactivity was suggested by molecular mechanics studies, which superimposed the optimized conformers of **1** and **2a**, and showed that the orientation of the 8-acetylamino groups differed by about 40° [2].

We now wish to report the synthesis of the isomers of **2a** (**2b**, **3a**, **b**) and the unsaturated analogue of **2b** (compound **2c**) (fig 1), which have been tested both *in vitro* and *in vivo* for their pharmacological properties.

Chemistry

As shown in scheme 1, compound **2b** was prepared from the commercially available 4-keto-4,5,6,7-tetrahydrothianaphthene **4**, which by treatment with bromine in acetic acid gave the corresponding 2-bromo derivative **5**. Nitration of **5** with potassium nitrate in sulfuric acid at 0°C led to **6**, which was dehalogenated

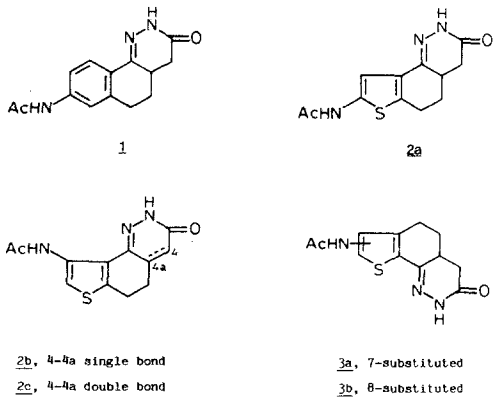

Fig 1. Structures of compounds **1**, **2a–c** and **3a**, **b**.

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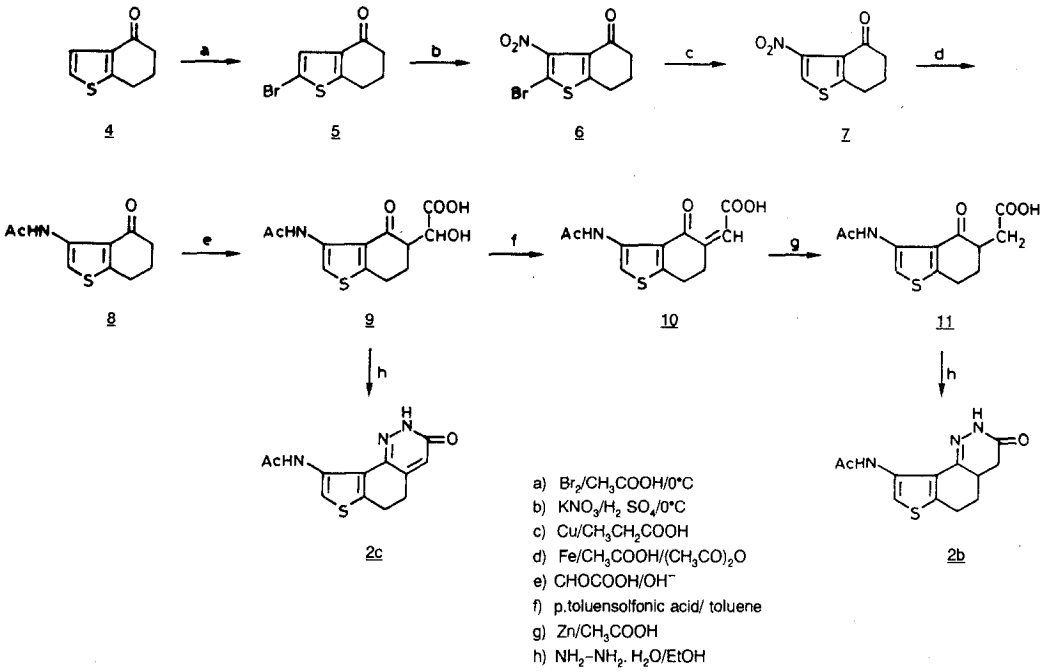


Scheme 1.

to **7** with copper in propionic acid. Reduction of **7** with iron in acetic acid/acetic anhydride at 80°C afforded the acetylamino derivative **8**, which was converted into the α-hydroxy acid **9** using glyoxylic acid. Dehydration of **9** with *p*-toluene sulfonic acid in toluene gave the unsaturated acid **10**, which by reduction with zinc in acetic acid was converted into **11**. Finally, cyclization with hydrazine hydrate in refluxing ethanol gave the desired 9-acetylamino-4,4*a*,5,6-tetrahydrothieno(2,3-*h*)cinnolin-3-(2*H*)-one **2b**. The corresponding 4,4*a*-dehydro derivative **2c** was obtained by directly condensing **9** with hydrazine hydrate in ethanol.

Compounds **3a**, **b** were synthesized following the method previously reported for isomer **2** [2], starting from the known 4,5,6,7-tetrahydro-7-oxobenzo(*h*)-thiophene **12** [3] (scheme 2). Accordingly, **12** was treated with bromine in dry ether to give the 6-bromo derivative **13**, which was reacted with ethyl malonate and sodium hydride in dry ether to give 43% of **14**. Nitration of **14** with potassium nitrate and sulfuric acid at 0°C gave a mixture of the 2- and 3-nitro derivatives (**15b**, **15a**), which were easily separated by silica-gel chromatography in a ratio 1:2.3. The attribution of the structures was made on the basis of their ^{1}H-NMR spectra. The spectrum of **15b** had a singlet at δ 7.68, which was attributed to H3, whereas that of **15a** had a singlet at δ 8.64 for H2. This is due to the deshielding effects of the sulfur and the nitro group. Treatment of **15a**, **b** with powdered iron in acetic acid/acetic anhydride, gave the acetylamino derivatives **16a**, **b**, which were hydrolysed to **17a**, **b** and cyclized with hydrazine hydrate to give the desired **3a**, **b**.

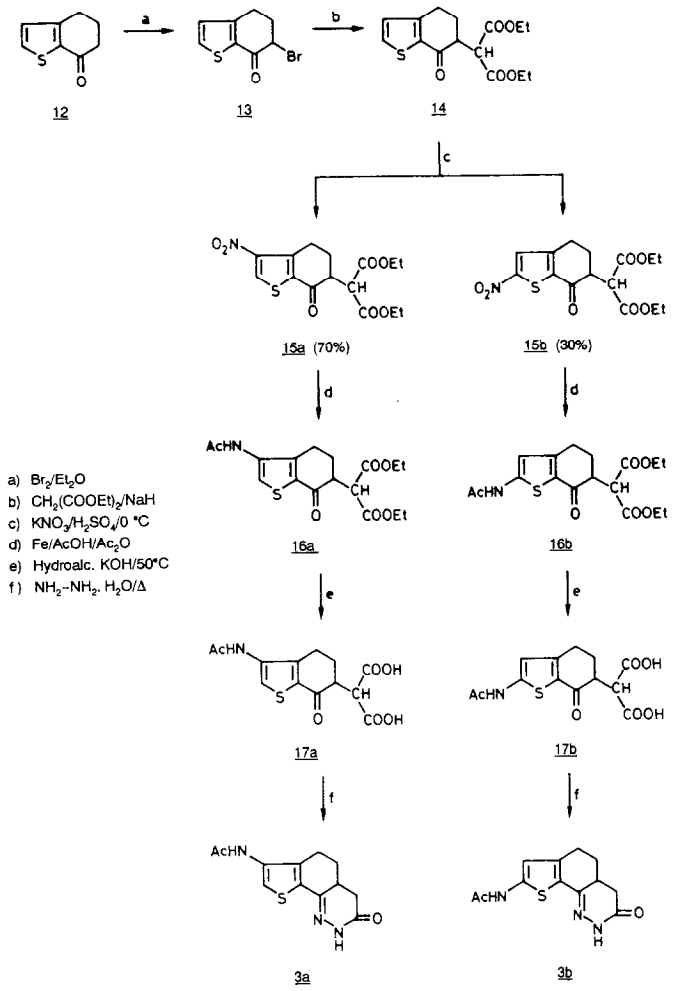
Pharmacology

Compounds **2b**, **c** and **3a**, **b** were tested for their hypotensive, antihypertensive and antiaggregating properties. Binding studies to assess their affinity to PDE isoenzymes were also performed. The benzocinnolinone **1** was used as a reference drug.

Results and discussion

Binding studies on PDE isoenzymes indicate that at 100 μm all the test compounds, with the exception of **2b**, exhibited a higher affinity towards the PDE III isoenzyme than **1**. However, in contrast to the model, which very weakly interacted with the other PDE isoenzymes, the new derivatives also showed some affinity towards PDE II (**2c** and **3b**) and PDE V (**2b**, **2c**) (table I).

In vivo experiments on spontaneously hypertensive rats (SHR) were carried out at 3 different doses (table II). At the highest dose of 25.0 mg/kg, only **3b** was



Scheme 2.

found to be comparable with the model **1**, while all the other derivatives were less active. However, it should be noted that, in contrast to previous reports on a series of cardiotonic dihydropyridazinones [4], aromatization of the pyridazinonic moiety of **2b** did not reduce the antihypertensive potency; the resulting **2c** had the same activity as **2b**, thus confirming the trend observed in the benzocinnolinonic series [1].

In normotensive rats, at an oral dose of 12.5 mg/kg, all the compounds were found to be less potent than **1** (table III).

Finally, the antiaggregating properties of the new compounds were determined in an *in vitro* test of collagen-induced plasma aggregation in human plasma. All compounds, with the exception of **2b**, displayed antiaggregating activities higher than the model at a final dose of 10^{-5} g/ml in the presence of 10^{-5} g/ml collagen (table IV).

The structure of the active compound **1** is consistent with a 5-point model proposed by Bristol [5] for cardiotonics acting through PDE III inhibition. It was thus of interest to verify to what extent compounds **2** and **3**, with the benzene ring replaced by a thiophene moiety, would fit the model.

Molecular mechanics calculations have thus been performed on compounds **2** and **3** with the program MM285 and according to the procedure previously described for **1** [6]. Considering the most stable conformations of **2** and **3** (fig 2), it can be observed that rings II and III completely superimpose on the corresponding rings of **1**. In contrast, rings I of the 3 structures have different geometrical features.

In fact, the orientation of the four C-H bonds in **1** could be described by an angle ranging from 0°, for C7-H, to 180° for C10-H. The hydrogen atom on C8 should therefore be described by an angle of about 60°. As previously reported, no C-H bond of **2** is superimposable on the C8-H bond of **1** [2]. In contrast, compound **3** presents orientation angles of 11° and 80° for C7-H and C8-H, respectively. Thus, the orientation of the acetylamino group of **3a** and **3b** differs by about 50° and 20°, respectively, from that of the C8-substituent in compound **1** (L Toma, personal communication).

Table I. Binding studies *via* inhibition of phosphodiesterase isoenzymes (PDE I–V)[a].

Compound	*% inhibition at 100 μM*					
	Va	*Ia*	*Id*	*II*	*III*	*IV*
1	19	3	11	26	54	7
2b	58	NI	25	39	33	27
2c	66	NI	34	65	68	15
3a	16	NI	9	NI	68	NI
3b	33	NI	NI	54	81	15

[a]For PDE preparation see *Experimental protocols*. NI: not inhibiting.

Table II. Antihypertensive activity.

Compound	*Dose mg/kg (po)*	*Systematic arterial blood pressure (mmHg ± se) (SHR)*						
		0 h[a]	*1 h*[a]	*2 h*[a]	*3 h*[a]	*4 h*[a]	*5 h*[a]	*6 h*[a]
1	6.2	190 ± 7.3	152 ± 17.2*	150 ± 16.5*	148 ± 18.1*	148 ± 14.1*	142 ± 15.6*	140 ± 13.2**
	12.5	188 ± 10.5	140 ± 17.7*	90 ± 18**	90 ± 14.7**	90 ± 13.6**	85 ± 17.4**	85 ± 12.9**
	25.0	191 ± 3.2	131 ± 10.8**	105 ± 15.4**	86 ± 12.2**	80 ± 16.4**	70 ± 13.5**	70 ± 9.9**
2b	6.2	181 ± 11.4	178 ± 10.8	170 ± 12.7	165 ± 11.9	157 ± 12.6	152 ± 11.5	150 ± 14.1
	12.5	179 ± 9.2	141 ± 11.9*	135 ± 14.8*	133 ± 13.3*	133 ± 14.4*	128 ± 15.4*	121 ± 16.2*
	25.0	184 ± 9.6	162 ± 3.2	156 ± 9.9*	151 ± 10.0*	148 ± 10.5*	136 ± 13.6**	122 ± 12.4**
2c	6.2	185 ± 9.7	158 ± 13.6	145 ± 19.6*	144 ± 19.4*	140 ± 12.9*	138 ± 14.1*	135 ± 17.4*
	12.5	177 ± 9.4	147 ± 9.0*	130 ± 9.2**	128 ± 8.3**	128 ± 9.1**	119 ± 10.2**	118 ± 9.4**
	25.0	189 ± 11.4	145 ± 12.5*	132 ± 10.7**	131 ± 12.8**	128 ± 13.6**	119 ± 9.9**	107 ± 13.1**
3a	6.2	185 ± 14.6	170 ± 18.8	165 ± 17.6	158 ± 19.6	158 ± 20.1	156 ± 18.7	156 ± 17.2
	12.5	200 ± 15.8	150 ± 14.9*	135 ± 18.8*	123 ± 17.4*	123 ± 19.2	122 ± 20.6	120 ± 18.2
	25.0	192 ± 12.5	144 ± 11.6*	118 ± 15.4**	114 ± 9.7**	114 ± 12.1**	108 ± 15.9**	103 ± 16.3**
3b	6.2	183 ± 7.7	159 ± 6.3*	146 ± 9.3*	133 ± 7.9**	126 ± 10.4**	119 ± 12.7**	115 ± 15.6**
	12.5	190 ± 17.3	157 ± 7.1*	125 ± 8.9**	103 ± 11.5**	103 ± 12.1**	101 ± 11.6**	100 ± 14.5**
	25.0	186 ± 11.2	132 ± 9.8**	108 ± 13.3**	82 ± 15.4**	82 ± 19.8**	70 ± 16.8**	68 ± 10.1**
Hydralazine	6.2	189 ± 9.2	156 ± 8.9*	149 ± 9.2*	126 ± 17.8**	126 ± 9.2**	119 ± 12.7**	115 ± 12.9**
	12.5	186 ± 9.3	141 ± 7.8**	128 ± 9.2**	124 ± 10.8**	120 ± 10.6**	116 ± 13.2**	106 ± 14.1**
	25.0	182 ± 9.5	138 ± 8.9**	122 ± 10**	121 ± 12.2**	114 ± 10.5**	115 ± 11.1**	110 ± 13.4**
Controls		181 ± 10.1	180 ± 9.7	183 ± 11.0	179 ± 10.3	182 ± 11.0	181 ± 9.9	180 ± 10.5

[a]Time elapsed after administration; *$P < 0.05$; **$P < 0.01$ *vs* basal values.

Table III. Hypotensive activity.

Compound	*Dose mg/kg (po)*	*Systemic arterial blood pressure (normotensive rats)*			
		0 h[a]	*1 h*[a]	*2 h*[a]	*3 h*[a]
1	6.2	140 ± 13	132 ± 14.5	110 ± 13.8	95 ± 22.6
	12.5	128 ± 13.2	70 ± 13.6*	40 ± 15.4**	40 ± 17.8**
2b	6.2	120 ± 11.3	116 ± 11.2	103 ± 7.3	98 ± 7.4
	12.5	124 ± 15.2	101 ± 10.2	89 ± 12.3	67 ± 5.4**
2c	6.2	125 ± 13.4	108 ± 9.7	105 ± 10	102 ± 9
	12.5	137 ± 10.6	87 ± 20.2*	70 ± 26*	60 ± 17.9**
3a	6.2	137 ± 15.8	123 ± 14.8	110 ± 11.0	112 ± 11.4
	12.5	134 ± 15.3	105 ± 9.3	95 ± 9.7*	95 ± 12.1*
3b	6.2	140 ± 10	112 ± 11.2*	106 ± 8.6*	104 ± 9.3*
	12.5	138 ± 9.3	60 ± 17.3**	60 ± 15.9**	65 ± 15.4**
Hydralazine	6.2	110 ± 8.1	90 ± 7.8	85 ± 8.4*	85 ± 7.6*
	12.5	122 ± 10.1	70 ± 8.3**	65 ± 9.6**	60 ± 11**
Controls		129 ± 11.9	130 ± 17.0	129 ± 11	128 ± 13.2

[a]Time elapsed after administration; *$P < 0.05$; **$P < 0.01$ *vs* basal values.

Table IV. Antiaggregating activity[a].

Compound	*Final concentration (g/ml)*	*% aggregation ± se*	*% vs collagen*
Collagen	10^{-5}	82.0 ± 1.7	–
ASA	10^{-6}	76.5 ± 0.9**	–6.7
	10^{-5}	65.0 ± 2.5**	–20.0
1	10^{-6}	76.2 ± 1.5*	–7.0
	10^{-5}	75.1 ± 2.1*	–8.4
2b	10^{-6}	81.0 ± 3.5	–1.2
	10^{-5}	78.5 ± 1.3	–4.2
2c	10^{-6}	79.5 ± 1.8	–3.0
	10^{-5}	69.0 ± 2.3	–15.8
3a	10^{-6}	72.5 ± 3.0*	–11.5
	10^{-5}	71.5 ± 3.6*	–12.8
3b	10^{-6}	71.8 ± 2.1*	–12.4
	10^{-5}	67.4 ± 3.2**	–17.8

[a]Platelet aggregation induced by collagen in human plasma. Mean value ± se of 5 determinations. *$P < 0.05$; **$P < 0.01$; *vs* collagen control.

From these data it could be inferred that an adequate orientation of the acetylamino group is one of the essential requirements for the activity of tricyclic pyridazinones and the lack of this requisite could be responsible for the inactivity of compounds **2a, b** and **3a**. Moreover, the interesting cardiovascular properties of **3b**, which superimposes on **1**, could be related, or at least partly, to PDE inhibition, as previously hypothesized for **1** [1]. In fact, although **3b** shows a relatively low potency against individual PDE isoenzymes, synergies could occur to produce a significant pharmacological effect.

Experimental protocols

Chemistry

Melting points (uncorrected) were determined with a Büchi 510 capillary apparatus. Analyses indicated by the symbols of the elements or functions were within ±0.4% of the theoretical values. IR spectra (nujol mull, unless otherwise noted) were recorded on a Perkin–Elmer 297 infrared spectrophotometer. UV spectra were recorded on a Perkin–Elmer Lambda 5 model. ^{1}H-NMR spectra were recorded on a Varian XL 200 with tetramethylsilane as the internal standard, using $CDCl_3$ as solvent, unless otherwise indicated.

4,5,6,7-Tetrahydro-2-bromo-4-oxobenzo(b)thiophene 5
To a solution of **4** (10 g; 0.066 mol) in 50% acetic acid (100 ml) cooled to –5°C, bromine (3.5 ml) in acetic acid (61.5 ml) was added dropwise and the mixture was stirred for 1 h. A solution of sodium acetate in water (30 ml) was then added and the so-formed precipitate filtered. Yield 90%; mp: 78–80°C (lit 78–79°C [7]).

4,5,6,7-Tetrahydro-2-bromo-3-nitro-4-oxobenzo(b)thiophene 6
To a solution of **5** (2 g; 0.008 mol) in sulfuric acid (14 ml) cooled to 0°C and under vigorous stirring, potassium nitrate was added in small portions so that the temperature never exceeded 5°C. The mixture was then stirred at 0°C for 0.5 h and poured onto ice. The so-formed yellow precipitate was filtered and crystallized from ethanol. Yield 87%; mp: 155–156°C (lit 156–157°C [8]).

4,5,6,7-Tetrahydro-3-nitro-4-oxobenzo(b)thiophene 7
To a solution of **6** (0.5 g; 0.0018 mol) in propionic acid (0.5 ml) vigorously stirred at 125°C, copper powder (0.5 g)

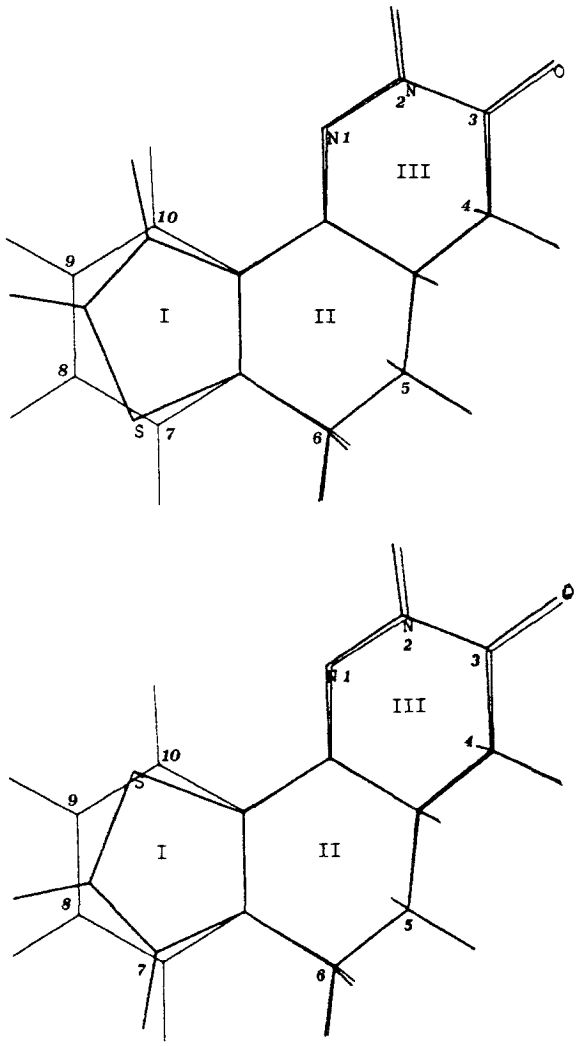


Fig 2. Superimposition of compounds **1** and **2** (top) and **1** and **3** (bottom).

was added portionwise. The mixture was further stirred for 5 min, cooled, mixed with silica gel and extracted in a Soxhelet with ethanol. The solvent was evaporated under vacuum, the residue washed in succession with sodium bicarbonate and water, and the insoluble product filtered off to give 0.25 g (71%) of **7**; mp: 120–121°C. IR: 1675 (CO); 1540, 1260 (NO_2) cm^{-1}. ^{1}H-NMR δ: 2.1–2.3 (m, 2H); 2.5–2.6 (m, 2H); 1 (t, 2H, J = 6 Hz); 8.1 (s, 1H). Anal $C_8H_7NO_3S$ (C, H, N, S).

*4,5,6,7-Tetrahydro-3-acetylamino-4-oxobenzo(b)thiophene **8***
To a solution of **7** (1 g; 0.005 mol) in acetic acid (20 ml) and acetic anhydride (4.8 ml) stirred at room temperature, iron (1.52 g; 0.027 mol) was added portionwise. The temperature was then slowly raised to 50°C and the mixture stirred for about 10 min until the reaction started. The temperature was then raised to 85°C and the mixture stirred for 10 h. After cooling, the mixture was poured onto water (80 ml), filtered and the residue washed with water and then chloroform. The aqueous layer was thoroughly extracted with chloroform. The reunited organic layers were dried over sodium sulfate, the solvent evaporated under vacuum and the solid residue triturated with water and filtered to give 0.8 g (75%) of **8**; mp: 88–90°C (ethanol). IR: 3300 (NH); 1680 (ketone CO); 1660 (amide CO) cm^{-1}. ^{1}H-NMR δ: 2.2–2.3 (m, 5H); 3.0 (t, 2H, J = 6 Hz); 3.1 (t, 2H, J = 6 Hz); 7.7 (s, 1H); 10.2 (br s, 1H, exchanges with D_2O). Anal $C_{10}H_{10}NO_2S$ (C, H, N, S).

*5(4,5,6,7-Tetrahydro-3-acetylaminobenzo(b)thiophene)-α-hydroxyacetic acid **9***
To a suspension of **8** (9 g; 0.04 mol) and glyoxylic acid (12.2 g; 0.1 mol) in water (162 ml) cooled to 0°C, a solution of sodium hydroxide (7.2 g; 0.18 mol) in water (320 ml) was added. The mixture was stirred for further 2 h at room temperature then acidified, under external cooling, with 6 N hydrochloric acid. The so-formed precipitate was filtered and crystallized from acetic acid to give 7.2 g (66%) of **9**; mp: 189–190°C. IR: 3500 (NH); 3300 (OH); 1720 (ketone CO); 1660 (acid CO); 1640 (amidic CO) cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 2.1–2.4 (m, 5H); 3.0–3.2 (m, 3H); 4.9 (br s, 2H, exchanges with D_2O); 5.0 (d, 1H); 7.6 (s, 1H); 10.3 (*s*, 1H, exchanges with D_2O). Anal $C_{12}H_{13}NSO_5$ (C, H, N, S).

*5(3-Acetylamino-4,5,6,7-tetrahydro-4-oxobenzo(b)thienylidene) acetic acid **10***
A mixture of **9** (1.0 g; 0.004 mol) and *p*-toluene sulfonic acid (0.7 g; 0.004 mol) in toluene (12 ml) was stirred at 100°C for 1.5 h. The solvent was removed and the residue purified by silica-gel chromatography (eluent chloroform) to give as the first run the desired **10**. Yield 35%; mp: 215–220°C. IR: 3320 (NH); 1710 (ketone CO); 1650 (acid CO; amidic CO) cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 2.1 (s, 3H); 3.1–3.2 (m, 2H); 3.3–3.5 (m, 2H); 4.0 (br s, 1H, exchanges with D_2O), 6.6 (s, 1H); 7.7 (s, 1H); 10.1 (br s, 1H, exchanges with D_2O). Anal $C_{12}H_{11}NO_4S$ (C, H, N, S).

*5(3-Acetylamino-4,5,6,7-tetrahydro-4-oxobenzo(b)thiophene) acetic acid **11***
A mixture of **10** (0.7 g; 0.0026 mol) and zinc dust (0.47 g; 0.0072 mol) in acetic acid (6.0 ml) and water (2.0 ml) was heated at 100°C for 40 min. After cooling, the solid was filtered off and thoroughly washed with water. The filtrate was extracted with dichloromethane, dried over sodium sulfate and the solvent evaporated to give 0.55 g (80%) of **11**, which was crystallized from ethanol; mp: 198–200°C. IR: 3400–3300 (NH, OH); 1720 (ketone CO), 1660 (acid CO, amidic CO) cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 2.1–2.2 (m, 5H); 2.4–2.7 (m, 2H); 2.9–3.1 (m, 6H); 7.6 (s, 1H); 10.1 (s, 1H, exchanges with D_2O); 12.2 (br s, 1H, exchanges with D_2O). Anal $C_{12}H_{13}NO_4S$ (C, H, N, S).

*9-Acetylamino-4,4a,5,6-tetrahydrothieno(2,3-h)cinnolin-3-(2H)-one **2b***
A solution of **11** (1 g; 0.0037 mol) and hydrazine hydrate (0.3 ml; 0.009 mol) in ethanol (26 ml) and few drops of acetic acid was refluxed for 24 h. The solid that formed after cooling was collected by filtration to give 0.7 g (75%) of **2b**; mp: 223–225°C (ethanol). IR: 3300 (NH); 1660 (amidic CO) cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.6–1.7 (m, 1H); 2.1 (s, 3H); 2.2–3.0 (m, 6H); 7.6 (s, 1H); 10.6 (s, 1H, exchanges with D_2O); 10.9 (s, 1H, exchanges with D_2O). Anal $C_{12}H_{13}N_3O_2S$ (C, H, N, S).

*9-Acetylamino-5,6-dihydrothieno(2,3-h)cinnolin-3-(2H)-one **2c***
A solution of **9** (1 g; 0.0037 mol) and hydrazine hydrate (0.54 ml) in ethanol (4 ml) was refluxed for 4 h. The solvent was evaporated, the residue treated with 5% sodium bicarbonate and the insoluble filtered and thoroughly washed with water to give, after drying at 50°C, 0.3 g (27%) of **2c**; mp 295–298°C (dec). IR: 3300 (NH); 1690, 1670 (CO) cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 2.1 (s, 3H); 3.0 (app s, 4H); 6.8 (s, 1H); 7.7 (s, 1H); 10.2 (s, 1H, exchanges with D_2O); 12.8 (s, 1H, exchanges with D_2O). Anal $C_{12}H_{11}N_3O_2S$ (C, H, N, S).

*4,5,6,7-Tetrahydro-6-bromobenzo(b)thiophen-7-one **13***
To a solution of **12** (3.75 g; 0.025 mol) in anhydrous ether (75 ml) cooled at 0°C, bromine (3.92 g; 0.039 mol) was added dropwise. The mixture was stirred at room temperature for 1 h and then poured onto ice and extracted with ether. The organic layer was washed with 5% $NaHCO_3$, dried over sodium sulfate and evaporated under vacuum to give 4.66 g (82%) of an oil that solidified after standing. The analytical sample was crystallized from petroleum ether; mp: 63–65°C. IR: 1640 (C=O) cm^{-1}. ^{1}H-NMR δ: 2.5–3.2 (m, 5H); 4.7 (t, 1H, J = 4 Hz); 7.3 (ABq, 2H). Anal C_8H_7BrOS (C, H, Br, S).

*4,5,6,7-Tetrahydro-7-oxobenzo(b)thiophene-6-malonic acid diethyl ester **14***
Ethyl malonate (3.0 g; 0.018 mol) is added dropwise under a slow stream of nitrogen to a suspension of NaH (50% in oil; 0.89 g; 0.085 mol) in dry THF (4 ml) at 0–5°C. A solution of **13** (3.7 g; 0.016 mol) in dry THF (19 ml) was then added and the mixture stirred for 2 h at room temperature. Water was added, the organic layer separated and the aqueous layer extracted twice with ether (2 x 20 ml). The combined organic layers were dried over sodium sulfate and the solvent was evaporated to give a residue that was purified by flash chromatography (eluent petroleum ether/ethyl acetate 8:2) to give 2.9 g (53%) of **14**, bp: 190°C/0.05 mmHg. IR: 1760, 1740 (ester C=O); 1670 (ketone C=O) cm^{-1}. UV (EtOH) λ_{max} nm (log ε): 271.6 (3.66); 199.3 (3.12). ^{1}H-NMR δ: 1.1–1.2 (m, 6H); 2.2–2.4 (m, 2H); 2.3–3.1 (m, 2H); 3.4 (m, 1H); 4.0 (d, 1H); 4.2–4.3 (m, 4H); 7.3 (ABq, 2H). Anal $C_{15}H_{18}O_5S$ (C, H, S).

*4,5,6,7-Tetrahydro-3-nitro-7-oxobenzo(b)thiophene-6-malonic acid diethyl ester **15a** and its 2-nitro analogue **15b***
To a stirred solution of **14** (1 g; 0.0032 mol) in concentrated sulfuric acid (10 ml) cooled at 0–3°C, powdered KNO_3 (0.8 g; 0.0079 mol) was added portionwise. After stirring at 0–3°C for 30 min the mixture was poured onto ice to give a yellow gummy product, which was extracted with ether. The organic layer was washed with water, dried over sodium sulfate and the solvent was evaporated to give 0.96 g (84%) of a mixture of 2 nitro derivatives, which were separated by silica-gel chro-

matography (eluent petroleum ether/ethyl acetate 8:2) to give in the order of elution 0.64 g (70%) of **15a** and 0.32 g (30%) of **15b**.

*Compound **15a***. Mp: 65–67°C (ethanol). IR: 1740, 1720 (ester C=O); 1650 (ketone C=O); 1540, 1340 (NO_2) cm^{-1}. UV (EtOH). λ_{max} nm (log ε): 258.0 (3.77). ^{1}H-NMR δ: 1.2–1.3 (m, 6H); 2.2–2.4 (m, 2H); 3.0–3.6 (m, 3H); 4.1 (d, 1H); 4.2–4.3 (m, 4H); 8.6 (s, 1H). Anal $C_{15}H_{17}NO_7S$ (C, H, N, S).

*Compound **15b***. Mp: 64–65°C (ethanol). IR: 1760, 1720 (ester C=O); 1670 (ketone C=O); 1520, 1340 (NO_2) cm^{-1}. ^{1}H-NMR δ: 1.1–1.3 (m, 6H); 2.1–2.2 (m, 2H); 2.8–3.5 (m, 3H); 4.0 (d, 1H); 4.1–4.4 (m, 3H); 7.7 (s, 1H). Anal $C_{15}H_{17}NO_7S$ (C, H, N, S).

*4,5,6,7-Tetrahydro-3-acetylamino-7-oxo-benzo(b)thiophene-6-malonic acid diethyl ester **16a***
To a solution of **15a** (2.62 g; 0.0074 mol) in acetic acid (37.0 ml) and acetic anhydride (8 ml), iron powder (2.27 g; 0.040 mol) was added and the mixture gradually heated to 80°C and then stirred for 10 h. The mixture was poured onto water and heated to 60°C. It was filtered when still hot and extracted with chloroform. The solvent was dried over sodium sulfate and evaporated to give an oil which spontaneously solidified on standing. Yield 69%; mp: 123–125°C (isopropyl ether). IR: 3300 (NH); 1750, 1730 (ester C=O); 1680 (ketone C=O); 1660 (amidic CO) cm^{-1}. ^{1}H-NMR δ: 1.2–1.8 (m, 6H); 2.2 (s, 3H); 2.2–2.4 (m, 2H); 2.7–2.8 (m, 2H); 3.2–3.4 (m, 1H); 4.0 (d, 1H); 4.1–4.3 (m, 4H); 7.2 (br s, 1H, exchange with D_2O); 8.0 (s, 1H). Anal $C_{17}H_{21}NO_6S$ (C, H, N, S).

*4,5,6,7-Tetrahydro-2-acetylamino-7-oxobenzo(b)thiophene-6-malonic acid diethyl ester **16b***
Compound **16b** was prepared as reported above for **16a** starting from **15b**. Yield 86%; mp: 173–176°C (toluene). IR: 3380 (NH); 1750 (ester CO); 1680 (ketone CO); 1660 (amidic CO) cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.2–1.3 (m, 6H); 2.2 (s, 3H); 2.1–2.4 (m, 2H); 2.6–2.9 (m, 2H); 3.3 (m, 1H); 4.0 (d, 1H); 4.1–4.3 (m, 4H); 6.5 (s, 1H); 9.6 (br s, 1H, exchange with D_2O). Anal $C_{17}H_{21}NO_6S$ (C, H, N, S).

*4,5,6,7-Tetrahydro-3-acetylamino-7-oxobenzo(b)thiophene-6-malonic acid **17a***
A solution of **16a** (1.7 g; 0.0048 mol) in ethanol (14 ml) and 2 N sodium hydroxide (6 ml) was heated at 50°C for 24 h. Water (4 ml) was then added and the mixture was concentrated under vacuum and extracted with ethyl acetate (2 x 10 ml). After acidification to pH 3 with 6 N hydrochloric acid the aqueous layer was re-extracted with ethyl acetate, the organic layer dried over sodium sulfate and evaporated to give 90% of crude **17a**, which was used as such for the next step.

*7-Acetylamino-4,4a,5,6-tetrahydrothieno(2,3-h)cinnolin-3-(2H)-one **3a***
A solution of crude **17a** (1.8 g; 0.006 mol) in hydrazine hydrate (5 ml) was refluxed for 30 min. After cooling, a solid precipitated. This was filtered, washed with water and crystallized from dimethylformamide. Yield 73%; mp: 325°C (dec). IR: 3280 (NH); 1680, 1650 (C=O) cm^{-1}. UV (EtOH) λ_{max} nm (log ε): 322.9 (3.5); 277.8 (3.6); 200.3 (3.4). ^{1}H-NMR δ: 2.1 (s, 3H); 2.1–3.0 (m, 7H); 7.6 (s, 1H); 9.4 (br s, 1H; exchange with D_2O); 10.8 (br s, 1H, exchange with D_2O). Anal $C_{12}H_{13}N_3O_2S$ (C, H, N, S).

*8-Acetylamino-4,4a,5,6-tetrahydrothieno(2,3-h)cinnolin-3-(2H)-one **3b***
Compound **3b** was prepared in 2 steps as reported above for **3a** starting from **16b**. Yield 71%. IR: 3240 (NH); 1660 (amidic CO) cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 2.0 (s, 3H); 2.1–3.0 (m, 7H); 7.8 (s, 1H); 10.3 (br s, 1H, exchange with D_2O); 11.0 (br s, 1H, exchanges with D_2O). Anal $C_{12}H_{13}N_3O_2S$ (C, H, N, S).

Pharmacology

Preparation of phosphodiesterase enzymes
The Ca^{2+}/calmodulin-stimulated PDE (PDE Ia; nomenclature as in reference [9]) was prepared from bovine cardiac ventricle. Following chromatography on a Mono Q column, the fractions showing stimulation of PDE activity by Ca^{2+} and calmodulin were pooled and further purified on a calmodulin-affinity column. PDE Id was prepared in a similar fashion from guinea-pig cardiac ventricle. cGMP-stimulated PDE (PDE II), cGMP-inhibited PDE (PDE III) and cAMP-specific PDE (PDE IV) were isolated from guinea-pig cardiac ventricle. Initial chromatography on a 20 ml Mono Q column resolved the PDE III from a peak that contained both PDE II and PDE IV. The latter peak was separated on a cGMP-affinity column [10]. The resolved PDEs were rechromatographed separately on a 1 ml Mono Q column. cGMP-selective PDE (PDE Va) was prepared from porcine lung using chromatography on DEAE-cellulose and Mono Q columns; a calmodulin-affinity column was used to remove residual PDE I activity. With the exception of PDE II, which displayed cooperativity, all the preparations showed simple Michaelis–Menton kinetics. The PDE also responded to modulators in a predictable fashion (*eg*, only PDE I was stimulated by Ca^{2+}/calmodulin and only PDE III was inhibited by cGMP).

Binding assay to PDE isoenzymes
PDE activity was assayed by the previously described boronate column method [11], using 1 μM cGMP as a substrate for PDE Ia, Id (in the absence of calmodulin), II and Va, and 1 μM cAMP as a substrate for PDE III and IV. Percentage inhibition of the test compounds at 100 μM was determined as the mean of 2 experiments carried out in duplicate.

Effects on arterial blood pressure
Male normotensive Sprague–Dawley rats (250–300 g) and spontaneously hypertensive rats (SHR, 250–300 g) were housed at constant temperature (21 ± 1°C) and relative humidity (60%) with free access to food and water. Animals were maintained under a regular 12 h light/dark schedule (light 7.00 am to 7.00 pm). Five animals were used for each experimental group. Arterial blood pressure of both unanaesthetized normotensive and spontaneously hypertensive rats was measured by the tail cuff method, utilizing a tail plethismographic apparatus LETICA LE 5000. The test compounds were suspended in 1% methylcellulose and administered in a volume of 10 mg/kg by oral route (po) at doses of 6.2, 12.5 and 25.0 mg/kg. Arterial blood pressure was recorded every hour for 6 h after drug administration. Hydralazine was taken as reference drug. Controls received the vehicle only. All results are expressed as mean ± standard error (se), with $P < 0.05$ and $P < 0.01$ considered as the significance level. Significant values were calculated in comparison with the basal values using Student's *t*-test.

Effects on platelet aggregation
Blood was collected from male and female donors who took no aspirin or any other drug influencing platelet aggregation.

Platelet rich plasma (PRP) and platelet poor plasma (PPP) were then prepared. PRP was obtained by centrifugating the blood samples for 15 min at 180 *g* at room temperature. The supernatant was removed and transferred to 10 ml volume plastic tubes. The remaining blood was further centrifuged for 15 min at 1200 *g* to get PPP. The PRP was adjusted with analogous PPP to 300 000 platelets/μl. Platelet aggregation was studied by Born's turbidimetric technique [12] on an Aggrecorder II PA 3220 aggregometer (Menarini Diagnostici, Florence, Italy) with collagen at a final concentration of 2 μg/ml. The platelet aggregation mixture was constituted of 450 μl of PRP and 50 μl of saline or test compound solution. Test compounds and acetylsalicylic acid (ASA) were diluted in saline and added to PRP samples to give a final concentration of 1 μg/ml and 10 μg/ml. The mixture was incubated at 37°C for 15 min before the addition of collagen, then the response was recorded for 5 min. Saline samples were always used first as a test to check whether platelets responded properly, and at the end of the experiment to make sure that PRP had not lost its responsiveness. The spontaneous platelet aggregation was also studied by placing the sample in the aggregometer and recording the light transmission for 5 min after the addition of both the test compounds and acetylsalicylic acid at 2 different concentrations. The aggregation response in the presence or absence of drugs was recorded as the peak height of the aggregation wave. The platelet aggregation inhibition percentage by test agents was calculated by the following formula:

$$\% \text{ inhibition} = \frac{\text{control aggregation} - \text{test aggregation}}{\text{control aggregation} \times 100}$$

All results are expressed as mean ± standard error (se) with $P < 0.05$ and $P < 0.01$ considered as the level of the significance. Significant values were calculated in respect to collagen using Student's *t*-test.

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