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Synthesis, antiplatelet and antithrombotic activities of new 2-substituted benzopyrano[4,3-*d*]pyrimidin-4-cycloamines and 4-amino/cycloamino-benzopyrano[4,3-*d*]pyrimidin-5-ones

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Abstract—Atherothrombotic coronary artery disease, associated with deep vein thrombosis, is one of the most common causes of death worldwide. Recently, antiplatelet combination therapy using agents with different mechanisms of action, such as aspirin, dipyridamole, and thienopyridines, seems to be an attractive preventive approach. Moreover, several large, randomized clinical trials support combination therapy with aspirin plus warfarin in high-risk patients with atherosclerotic heart disease. Our research on the benzopyrano[4,3-*d*]pyrimidine system gave rise to the synthesis of a large number of compounds endowed with in vitro anti-aggregating activity. Several SAR considerations suggest that the benzopyranopyrimidine system is an appropriate scaffold to obtain molecules that are able to act simultaneously in different pathways of aggregation. Now, we report the synthesis of new 2-substituted benzopyrano[4,3-*d*]pyrimidin-4-cycloamines and 4-amino/cycloamino-benzopyrano[4,3-*d*]pyrimidin-5-ones and the results of the pharmacological study on haemostasis. Some tested compounds showed a large-spectrum antiplatelet activity in vitro, and are more potent than aspirin as antithrombotics in vivo but, at variance with aspirin, they do not increase bleeding. This paper describes novel antithrombotic compounds with an interesting pharmacological profile and a potentially attractive benefit/risk ratio, with their mechanism of action generally, but not exclusively, dependent on antiplatelet activity, deserving further investigations.

1. Introduction

Atherothrombotic coronary artery disease, giving rise to a number of cardiocirculatory disorders, such as myocardial infarction (MI), unstable angina (UA), or acute stroke associated with deep vein thrombosis (DVT), is one of the most common causes of death worldwide and a growing public health problem.[1] To prevent further ischaemic events and vascular death ensuing from thrombosis, primary and secondary prevention strategies are currently adopted for patients with cardiovascular diseases.[2]

Thrombosis may occur when the haemostatic stimulus becomes unregulated. Important predisposing conditions to thrombosis are disturbed blood flow, hypercoagulation, and altered vessel wall.[3,4] Arterial thrombi are predominantly composed of platelets, a small amount of fibrin and a few red blood cells. It is for this reason that antiplatelet agents are successfully used in the treatment and prevention of arterial thrombosis.[5] Venous thrombi are mainly composed of red blood cells in a fibrin mesh, and anticoagulant agents are used in the treatment of venous thrombosis.

The relevance of antiplatelet drugs has been firmly established by clinical trials and experience with drugs, such as aspirin, dipyridamole and the thienopyridines.[6–8] These drugs are the only oral antiplatelet agents currently approved by the FDA (Food and Drug Administration) for use in patients. Recently, antiplatelet combination therapy using agents with different

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mechanisms of action seems to be an attractive preventive approach, since different signalling pathways contribute to platelet activation.[9,10] In this way, several trials with clopidogrel plus aspirin, including CURE (Clopidogrel in Unstable angina to prevent Recurrent Events),[11] MATCH (Management of Atherothrombosis with Clopidogrel in High-Risk Patients with Recent Transient Ischaemic Attacks or Ischemic Stroke),[12] and CHARISMA (Clopidogrel for High Atherothrombotic Risk and Ischaemic Stabilization, Management and Avoidance),[13] demonstrated the benefit of the combination therapy.[14]

Moreover, in spite of reluctance to use oral anticoagulants, several large, randomized clinical trials support combination therapy with aspirin plus warfarin in high-risk patients with atherosclerotic heart disease.[15,16] The WATCH (Warfarin and Antiplatelet Therapy in Chronic Heart Failure) trial was undertaken to determine the optimal antithrombotic therapy for heart failure patients.[17]

In the last few years, our research on the benzopyrano[4,3-*d*]pyrimidine system gave rise to the synthesis of a large number of compounds (shown in structure **1**, Fig. 1) endowed with interesting pharmacological properties such as antiinflammatory, analgesic and, particularly, in vitro anti-aggregating activities.[18–22]

The large number of data obtained in this pharmacological screening allowed us to make useful SAR predictions. In fact, several lines of evidence have confirmed that antiplatelet activity was dependent on concomitant contribution of both functions inserted in positions 2 and 5 of the heterocyclic system. Inhibition of ADP-induced platelet aggregation, for instance, is present in 2-methylthio-5-amino derivatives or in 2-methoxy-5-alkoxy/phenoxy derivatives, but neither in 2-methoxy-5-amino nor in 2-methylthio-5-alkoxy/phenoxy ones.[22] On the contrary, no chemical modification affects antiplatelet activity against arachidonic acid (AA)-induced aggregation: in fact, most of compounds **1**, apart from the substituents introduced, are active as antiplatelets, frequently as potent as acetyl salicylic acid (ASA). Moreover, the 2,5-cycloamino disubstituted compounds showed a large spectrum of action, inhibiting ADP-, AA-, and collagen-induced aggregation. Some of these were, in vivo, more potent antithrombotics than lysine acetylsalicylate and showed prohaemorrhagic activity lower than that of the reference drug.[23] The last synthesized 2-methanesulfonyl-5-alkoxy derivatives showed a remarkable in vitro antiplatelet activity of broad spectrum, being active against ADP-, AA-, and U46619-induced aggregation.[22] These results convinced us that the benzopyranopyrimidine system is an appropriate scaffold to obtain molecules that are able to act simultaneously in the different intracellular pathways that are involved in platelet aggregation and to serve as potent and effective antiplatelet agents.

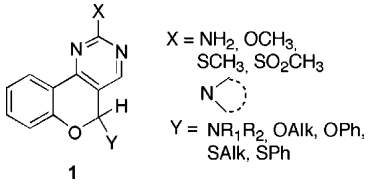

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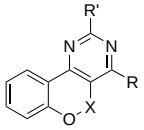
Figure 1. General structure of compounds **1**.

For the above-mentioned reasons, and according to the observation that a large spectrum of antiplatelet activity is obtained when a small, oxygen function is present in position 5 of the benzopyranopyrimidine system, we planned the synthesis of the new molecules **2**–**5** (Fig. 2, Table 1) in which a carbonyl function has been inserted in position 5 of the benzopyrano moiety.

Furthermore, in these new derivatives we achieved the fusion of pyrimidine cycle with the benzopyran-2-one system, well-known as the typical structure of anticoagulants drugs. The anticoagulant activity of coumarins, blocking the prothrombin biosynthesis by inhibition of vitamin K-epoxide reductase, is closely dependent on the presence of a hydroxy group in position 4 and a highly lipophilic substituent in position 3 of the benzopyrano (see Warfarin). On the other hand, despite the lack of aforementioned structural requirements, a similar mechanism of action to coumarin is supposed to take place for the anticoagulant 1,3-indandione derivatives (see Miradon).

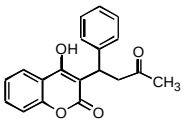


2-7

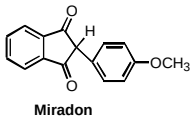
Figure 2. General structure of compounds **2**–**7**.

Table 1. Compounds **2**–**7**

Compound	X	R	R′
2a	C=O	H	OCH_3
2b	C=O	H	SCH_3
2c	C=O	H	Pyrrolidino
2d	C=O	H	Morpholino
3a	C=O	NH_2	H
3b	C=O	NH_2	NH_2
3c	C=O	NH_2	OCH_3
3d	C=O	NH_2	SCH_3
3e	C=O	NH_2	Pyrrolidino
3f	C=O	NH_2	Morpholino
4a	C=O	Pyrrolidino	SCH_3
4b	C=O	Piperidino	SCH_3
4c	C=O	Morpholino	SCH_3
5a	C=O	Pyrrolidino	SO_2CH_3
5b	C=O	Piperidino	SO_2CH_3
5c	C=O	Morpholino	SO_2CH_3
6a	CH_2	Pyrrolidino	SCH_3
6b	CH_2	Piperidino	SCH_3
6c	CH_2	Morpholino	SCH_3
7a	CH_2	Pyrrolidino	SO_2CH_3
7b	CH_2	Piperidino	SO_2CH_3
7c	CH_2	Morpholino	SO_2CH_3

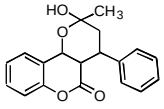


Warfarin



Miradon

The tricyclic structure of our new benzopyrano[4,3-*d*]pyrimidin-5-one derivative could mimic the hemichetal coumarin's active form (see the hemichetal of Warfarin).



Warfarin: hemichetal structure

As regards the position 2 of compounds **2**–**5**, we maintained the same substituents that were essential for antiplatelet activity in compounds **1**. Moreover, in position 4, which was never substituted in previous compounds, we inserted NH_2 and cycloamines with the aim of obtaining new information for SAR studies.

Finally, reduction of carbonyl in position 5 of compounds **4**,**5** yielded the 2-methylthio- and 2-methanesulfonyl-4-cycloamino derivatives **6**–**7**, already planned, but never obtained, by other alternative synthetic ways.[22]

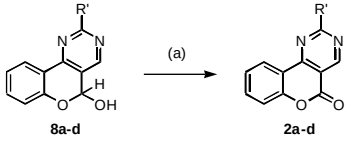
Hence, in this work we describe the synthesis and activity on haemostatic processes of compounds **2**–**7**.

2. Chemistry

2-Substituted benzopyrano[4,3-*d*]pyrimidin-5-ones **2a**–**d** were prepared from the suitable hemiacetal derivatives **8a**–**d**[19,21,22] by oxidation with $KMnO_4$ in alkali, as already described by Petersen and Heitzer[24] (see Scheme 1).

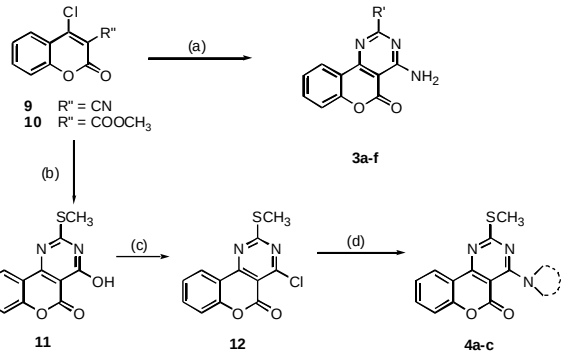
Starting from 3-cyano-4-chlorocoumarin **9**,[25] with an excess of the suitable amidine or guanidine in dry ethanol at 40 °C for 4 h, we obtained the 2-substituted 4-amino-benzopyrano[4,3-*d*]pyrimidin-5-ones **3a**–**f** (see Scheme 2). Compounds **2b**,**c**, and **3b**, yet synthesized by Petersen and Heitzer[24] and Checchi et al.,[25] respectively, were never tested for pharmacological activities.

4-Cycloamino-2-methylthio-benzopyrano[4,3-*d*]pyrimidin-5-ones **4a**–**c** were obtained as follows: methyl ester

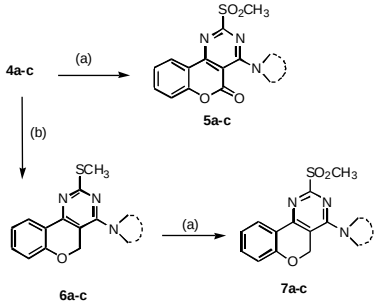


8a-d 2a-d

Scheme 1. Synthesis of compounds **2a**–**d**: (a) $KMnO_4$, NaOH, H_2O, reflux, 2 h.



Scheme 2. Synthesis of compounds **3a**–**f** and **4a**–**c**: (a) Compound **9**, amidine or guanidine, EtOH, 40 °C, 4 h; (b) Compound **10**, *S*-methylisothiourea, EtOH, 60 °C, 3 h; (c) $POCl_3$, 150 °C, 5 h; (d) Cycloamine, dry toluene, reflux, 3 h.



Scheme 3. Synthesis of compounds **5a**–**c**, **6a**–**c**, and **7a**–**c**: (a) *m*-chloroperbenzoic acid, $CHCl_3$, r.t., 12 h; (b) $NaBH_4$, BF_3/Et_2O; diglyme, dry THF, reflux, 3 h.

of 4-chloro-2-oxo-2*H*-[1]benzopyrano-3-carboxylic acid **10**[26] reacted with an excess of *S*-methylisothiourea in dry ethanol to give compound **11** which, by chlorination with an excess of $POCl_3$, afforded the intermediate **12**. Then, replacement of chlorine with suitable amine, in dry toluene, gave the desired compounds **4a**–**c** (see Scheme 2). Subsequent oxidation of SCH_3 group of compounds **4a**–**c**, with an excess of *m*-Cl-perbenzoic acid in $CHCl_3$ at room temperature, yielded 2-methanesulfonyl analogues **5a**–**c** (see Scheme 3).

(2-Methylthio-benzopyrano[4,3-*d*]pyrimidino)-4-cycloamines **6a**–**c** were prepared from compounds **4a**–**c** by reduction of the carbonyl with sodium borohydride, boron trifluoride diethyl etherate, and diglyme in dry THF at reflux [27] (see Scheme 3).

Finally, by oxidation of compounds **6a**–**c**, following the same procedure reported above, we obtained the 2-methanesulfonyl derivatives **7a**–**c** (see Scheme 3).

3. Pharmacology

The new compounds were studied in vitro towards arachidonic acid- (AA), ADP- and the stable TXA_2-receptor agonist U46619-induced platelet aggregation in rabbit platelet rich plasma.

Furthermore, they were investigated against thrombin-induced clot retraction in rat platelet-rich plasma. Clot retraction is a physiological event that stabilizes the coagulum. It is triggered by an interaction between extracellular fibrinogen or fibrin and the activated fibrinogen receptor αIIbβ3, located on the platelet surface and anchored to intracellular actin and myosin chains. The ensuing contraction of actin/myosin filaments, within the platelet, places tension on the fibrin clot, causing it to contract.[28]

Both inhibition of platelet aggregation and clot retraction are critical factors for the activity of antithrombotic drugs. Antiplatelet agents limit the activation of platelets, which are essential elements to prime haemostatic plug formation; agents inhibiting retraction destabilize clots making them weaker and more likely to be degraded by the fibrinolytic system.[29]

Additionally, we investigated the in vivo antithrombotic effect produced by subacute treatment using an experimental model of pulmonary acute thromboembolism produced by collagen plus epinephrine injection in mice.[30]

Tolerability of the subacute treatment with such compounds was examined by evaluating their adverse pro-haemorrhagic effect in tail bleeding[31] and daily monitoring the body weight, behaviour, and physical appearance of the treated mice.

4. Results and discussion

Only a minority of new compounds inhibit platelet aggregation and clot retraction in vitro, as given in Table 2. This finding suggests that the presence of the carbonyl group in position 5 of the benzopyranopirimidine system and the simultaneous insertion of any substituents (except for SO_2CH_3) in positions 2 and 4 are detrimental to antiplatelet activity and for the prevention of the fibrin clotting retraction (compounds **2a–d**, **3a–f**, and **4a–c** became completely inactive up to 1 mM concentration) (Table 2).

Table 2. In vitro antiplatelet potency of compounds against arachidonic acid (AA), U46619, and ADP induced aggregation in rabbit PRP and inhibitory potency against thrombin induced clot retraction in rat PRP

Compounds	Inhibition of platelet aggregation IC_{50} (μM)			Inhibition of clot retraction IC_{50} (μM)
	ADP 3 μM	AA 50 μM	U46619 1 μM	Thrombin 20 U/mL
2a–d	—	—	—	—
3a–f	—	—	—	—
4a–c	—	—	—	—
5a	13	15	4	13
5b	18	16	21	27
5c	16	21	3	37
6a	—	45	—	—
6b	—	23	—	—
6c	—	71	—	—
7a	45	14	24	72
7b	>500	377	390	—
7c	—	>500	—	—
SQ 29548	>10	2	0.01	—
ASA	>500	61	—	—

The IC_{50} values (μM) are calculated from $n = 6–8$ distinct experiments.

In 2-SCH_3 derivatives **6a–c**, devoid of the carbonyl in position 5, translation of the cycloamine groups from position 5 to position 4 triggers a loss of antiplatelet activity against ADP, as a evidenced by comparison with compounds **1** (X = SCH_3, Y = NR_1R_2)[19] that have been previously described as potent and effective anti-aggregant agents against several aggregating substances. Therefore, among the tested compounds, only derivatives **6a–c** behave as selective inhibitors of AA-induced aggregation and show an ASA-like profile with similar antiplatelet potency and complete ineffectiveness in clot retraction.

The simultaneous presence of SO_2CH_3 in position 2 of benzopyranopyrimidinone nucleus and cycloamine in position 4 (obtained in derivatives **5a–c**) seems to represent a favourable structural feature to confer to the molecules a large-spectrum antiplatelet activity. The absence of carbonyl group in position 5 is well-tolerated only in the case of 2-pyrrolidinyl derivative (**7a**), which was as potent as compounds **5a–c** both in inhibiting platelet aggregation and clot retraction.

The compounds were studied in vivo by assessing their ability to prevent the paralysis caused by pulmonary acute thromboembolism in mice. After subacute intraperitoneal treatment, only compounds **2d**, **5a–c**, **6a**, and **7a** significantly protected mice from thromboembolic paralysis and they were more potent than aspirin but less powerful than the conventional anticoagulant warfarin (Fig. 3). Among these compounds, the 2-methanesulfonyl derivatives, equipotent in vitro as broad spectrum antiplatelet agents, displayed a slightly different antithrombotic potency in vivo (compound **5c** being three times more potent than compounds **5a**, **5b**, and **7a**). Moreover, a strong protective effect in vivo was also detected for 2-morpholinyl benzopyranopyrimidinone **2d** and 2-methylthio benzopyranopyrimidine **6a**, the former devoid of any antiplatelet activity and the latter active only towards AA-induced aggregation. Different explanations can account for the discrepancies between in vivo antithrombotic activity and in vitro antiplatelet property observed for the aforementioned compounds. Indeed, it is well-known that antithrombotic activity can be independent of the inhibition of platelet aggregation and that it can ensue from compounds, bioactivation or involvement of indirect mechanisms occurring only in vivo. Paradigmatic example is warfarin, which is an effective antithrombotic agent in vivo, devoid of any antiplatelet activity in vitro. At doses that are able to halve the percentage of thrombotic events compared to control group, the compounds do not produce any adverse side effects. Indeed, they do not increase bleeding at variance with aspirin (Fig. 4) nor do they affect the body weight and physical appearance of the treated mice (data not shown). Furthermore, they do not cause lethal events, as warfarin does, at antithrombotic doses

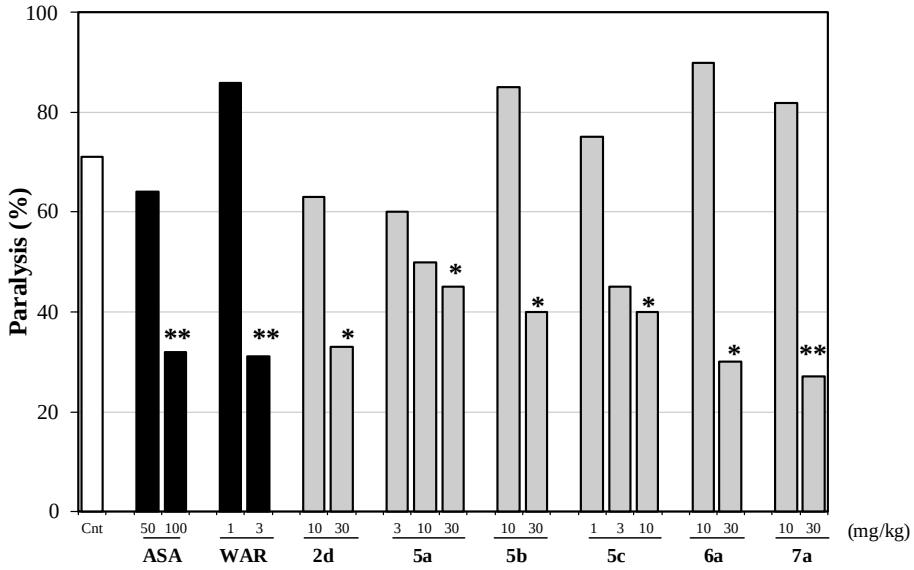


Figure 3. Acute pulmonary thromboembolism induced by i.v. shot injection of a mixture of 12 mg/kg collagen and 1 mg/kg epinephrine in mice, treated intraperitoneally for 5 days with vehicle alone (Cnt), the reference drugs aspirin (ASA) and warfarin (WAR) and the test compounds. Every column represents the percentage of paralysed animals. $*p < 0.05$ and $**p < 0.01$ by χ^2 test compared to control.

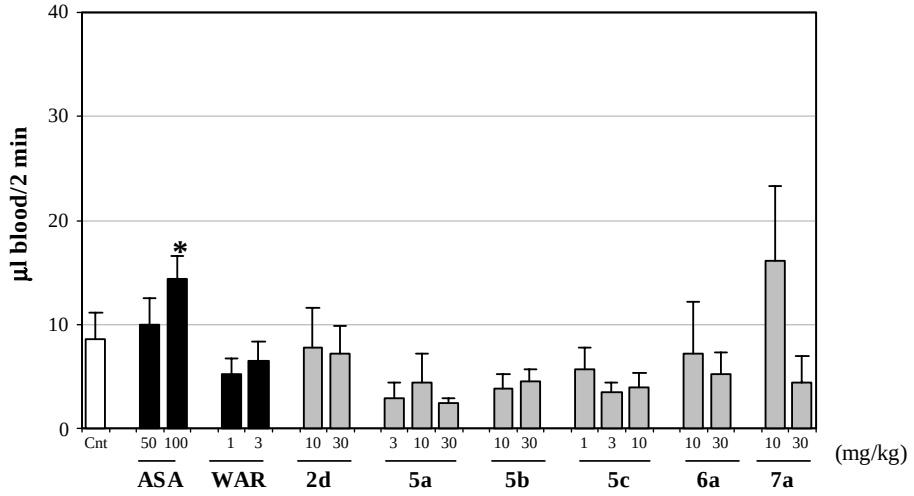


Figure 4. Bleeding test in mice treated intraperitoneally for 5 days with vehicle alone (Cnt), the reference drugs aspirin (ASA), and warfarin (WAR) and the test compounds. The volume of blood lost in 2 min by each mouse is reported as µL/2 min. $*p < 0.05$ by Mann–Whitney test.

(data not shown). This favourable risk benefit ratio increases the interest in these compounds whose antithrombotic activity, generally but not exclusively dependent on antiplatelet activity, deserves further investigations.

In conclusion, we synthesized several new benzopyrano[4,3-*d*]pyrimidine and benzopyrano[4,3-*d*]pyrimidin-5-one derivatives, some of them showing a large-spectrum antiplatelet activity in vitro and potency higher than that of aspirin as antithrombotics in vivo. Interestingly, the most active compounds seem to possess a potentially good benefit-risk profile; therefore, new pharmacological studies have been planned with the aim of examining their mechanism of action carefully.

5. Experimental protocols

5.1. Chemistry

All chemicals were obtained from Sigma–Aldrich s.r.l. (Milan, Italy).

Melting points are uncorrected and were measured with a Büchi 540 instrument. IR spectra were recorded with a Perkin-Elmer 398 spectrophotometer. ^{1}H NMR spectra were recorded on a Varian Gemini 200 (200 MHz) instrument; chemical shifts are reported as δ (ppm) relative to tetramethylsilane (TMS) as internal stardard; signals were characterized as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet) and br s (broad signal); J in Hz.

All compounds were tested for purity by TLC (Kieselgel 60F254 DC-Alufolien, E. Merck, Darmstadt, Germany). Analyses, indicated by the symbols of the elements or functions, were within ±0.4% of the theoretical values and were determined with an Elemental Analyzer EA 1110 (Fison-Instruments, Milan, Italy).

5.1.1. General procedure for 2-substituted 5*H*-[1]benzopyrano[4,3-*d*]pyrimidin-5-ones 2a–d. To a suitable 2-substituted 5-hydroxy-5*H*-[1]benzopyrano[4,3-*d*]pyrimidine **8** (10 mmol) were added NaOH (12.5 mmol, 0.5 g), $KMnO_4$ (10 mmol, 1.6 g) and H_2O (30 mL) and the mixture was stirred at reflux for 2 h. After cooling, the brown reaction mixture was filtered and the solution was acidified with conc. HCl (1.7 mL). The yellow solid obtained was filtered and recrystallized from DMF/H_2O (1:1) (compounds **2a**–**c**) or absolute ethanol (compound **2d**).

5.1.2. 2-Methoxy-5*H*-[1]benzopyrano[4,3-*d*]pyrimidin-5-one 2a. Mp 164–165 °C; yield: 31%. IR (KBr) cm^{-1}: 1734 (CO). ^1H NMR (DMSO-d_6) δ: 4.15 (s, 3H, OCH_3), 7.42–7.56 (m, 2H, H_7+H_8), 7.81 (t, $J = 6$, 1H, H_9), 8.42 (d, $J = 6$, 1H, H_{10}), 9.31 (s, 1H, H_4). Anal. Calcd for $C_{12}H_8N_2O_3$: C, 63.16; H, 3.53; N, 12.28. Found: C, 63.07; H, 3.61; N, 12.32.

5.1.3. 2-Methylthio-5*H*-[1]benzopyrano[4,3-*d*]pyrimidin-5-one 2b. Mp 187–188 °C (lit. 187–190); yield: 37% (lit. 27%). IR (KBr) cm^{-1}: 1725 (CO). ^1H NMR (DMSO-d_6) δ: 2.69 (s, 3H, SCH_3), 7.40–7.51 (m, 2H, H_7+H_8), 7.76 (t, $J = 6$, 1H, H_9), 8.40 (d, $J = 6$, 1H, H_{10}), 9.20 (s, 1H, H_4). Anal. Calcd for $C_{12}H_8N_2O_2S$: C, 59.01; H, 3.30; N, 11.47. Found: C, 59.25; H, 3.52; N, 11.20.

5.1.4. 2-Pyrrolidino-5*H*-[1]benzopyrano[4,3-*d*]pyrimidin-5-one 2c. Mp 205–206 °C (lit. 205–207); yield: 45% (lit. 45%). IR (KBr) cm^{-1}: 1726 (CO). ^1H NMR (DMSO-d_6) δ: 1.91–2.04 (m, 4H, $2CH_2$ pyrr), 3.30 and 3.65 (2m, 4H, $2CH_2N$ pyrr), 7.31–7.45 (m, 2H, H_7+H_8), 7.69 (t, $J = 6$, 1H, H_9), 8.31 (d, $J = 6$, 1H, H_{10}), 8.96 (s, 1H, H_4). Anal. Calcd for $C_{15}H_{13}N_3O_2$: C, 67.41; H, 4.90; N, 15.72. Found: C, 67.25; H, 4.80; N, 15,83.

5.1.5. 2-Morpholino-5*H*-[1]benzopyrano[4,3-*d*]pyrimidin-5-one 2d. Mp 195–196 °C; yield: 42%. IR (KBr) cm^{-1}: 1726 (CO). ^1H NMR (DMSO-d_6) δ: 3.69–3.87 (m, 4H, $2CH_2N$ morph), 3.92–4.10 (m, 4H, $2CH_2O$ morph), 7.38–7.45 (m, 2H, H_7+H_8), 7.76 (t, $J = 6$, 1H, H_9), 8.39 (d, $J = 6$, 1H, H_{10}), 9.04 (s, 1H, H_4). Anal. Calcd for $C_{15}H_{13}N_3O_3$: C, 63.60; H, 4.63; N, 14.83. Found: C, 63.24; H, 5.03; N, 14.50.

5.1.6. General procedure for 2-substituted 4-amino-5*H*-[1]benzopyrano[4,3-*d*]pyrimidin-5-ones 3a–f. To a solution of 4-chloro-3-cyanocoumarin **9** (5 mmol, 1.03 g) in absolute ethanol (10 mL) was slowly added a solution of suitable guanidine or amidine (obtained from the corresponding hydrochloride or hydrogen sulfate with equimolar EtONa) (10 mmol) in absolute ethanol and the mixture was stirred at 40 °C for 4 h. The solid obtained was filtered and recrystallized from DMF/H_2O (1:1).

5.1.7. 4-Amino-5*H*-[1]benzopyrano[4,3-*d*]pyrimidin-5-one 3a. Mp 287–288 °C; yield: 60%. IR (KBr) cm^{-1}: 3397, 3280 (NH_2), 1704 (CO). ^1H NMR (DMSO-d_6) δ: 3.36 (s, cryst. H_2O), 7.42–7.53 (m, 2H, H_7+H_8), 7.76 (t, $J = 6$, 1H, H_9), 8.22 (br s, 1H, NH disappears with D_2O), 8.41 (d, $J = 6$, 1H, H_{10}), 8.63 (br s, 1H, NH disappears with D_2O), 8.72 (s, 1H, H_2). Anal. Calcd for $C_{11}H_7N_3O_2$1/3H_2O: C, 60.27; H, 3.53; N, 19.17. Found: C, 59.96; H, 3.42; N, 19.50.

5.1.8. 2,4-Diamino-5*H*-[1]benzopyrano[4,3-*d*]pyrimidin-5-one 3b. Mp 288–290 °C (lit. 280–282); yield: 54%. IR (KBr) cm^{-1}: 3609, 3412, 3300 (NH_2), 1688 (CO). ^1H NMR (DMSO-d_6) δ: 7.22 (br s, 2H, NH_2, disappears with D_2O), 7.36–7.43 (m, 2H, H_7+H_8), 7.69 (t, $J = 6$, 1H, H_9), 7.83 (br s, 1H, NH, disappears with D_2O), 7.92 (br s, 1H, NH disappears with D_2O), 8.26 (d, $J = 6$, 1H, H_{10}). Anal. Calcd for $C_{11}H_8N_4O_2$: C, 57.89; H, 3.53; N, 24.55. Found: C, 57.69; H, 3.79; N, 24.66.

5.1.9. 2-Methoxy-4-amino-5*H*-[1]benzopyrano[4,3-*d*]pyrimidin-5-one 3c. Mp 277–278 °C; yield: 53%. IR (KBr) cm^{-1}: 3410, 3340 (NH_2), 1710 (CO). ^1H NMR (DMSO-d_6) δ: 3.34 (s, cryst. H_2O), 3.99 (s, 3H, OCH_3), 7.40–7.55 (m, 2H, H_7+H_8), 7.75 (t, $J = 6$, 1H, H_9), 8.20 (br s, 1H, NH, disappears with D_2O), 8.34 (d, $J = 6$, 1H, H_{10}), 8.58 (br s, 1H, NH, disappears with D_2O). Anal. Calcd for $C_{12}H_9N_3O_3$1/3H_2O: C, 57.83; H, 3.91; N, 16.86. Found: C, 58.02; H, 3.61; N, 16.94.

5.1.10. 2-Methylthio-4-amino-5*H*-[1]benzopyrano[4,3-*d*]pyrimidin-5-one 3d. Mp 278–280 °C; yield: 43%. IR (KBr) cm^{-1}: 3400, 3270 (NH_2), 1710 (CO). ^1H NMR (DMSO-d_6) δ: 2.60 (s, 3H, SCH_3), 3.35 (s, cryst. H_2O), 7.41–7.48 (m, 2H, H_7+H_8), 7.75 (t, $J = 6$, 1H, H_9), 8.17 (br s, 1H, NH, disappears with D_2O), 8.36 (d, $J = 6$, 1H, H_{10}), 8.58 (br s, 1H, NH, disappears with D_2O). Anal. Calcd for $C_{12}H_9N_3O_2S$.1/3H_2O: C, 54.33; H, 3.67; N, 15.84. Found: C, 54.25; H, 3.32; 15.80.

5.1.11. 2-Pyrrolidino-4-amino-5*H*-[1]benzopyrano[4,3-*d*]pyrimidin-5-one 3e. Mp 253–254 °C; yield: 59%. IR (KBr) cm^{-1}: 3501, 3375 (NH_2), 1677 (CO). ^1H NMR (DMSO-d_6) δ: 1.91–2.05 (m, 4H, $2CH_2$ pyrr), 3.38 (s, cryst. H_2O), 3.63–3.76 (m, 4H, $2CH_2N$ pyrr), 7.33–7.43 (m, 2H, H_7+H_8), 7.68 (t, $J = 6$, 1H, H_9), 7.90 (br s, 2H, NH_2, disappears with D_2O), 8.30 (d, $J = 6$, 1H, H_{10}). Anal. Calcd for $C_{15}H_{14}N_4O_2$.1/3H_2O: C, 62.49; H, 5.13; N, 19.43. Found: C, 62.68; H, 4.88; N, 19.73.

5.1.12. 2-Morpholino-4-amino-5*H*-[1]benzopyrano[4,3-*d*]pyrimidin-5-one 3f. Mp 229–230 °C; yield: 55%. IR (KBr) cm^{-1}: 3438, 3314 (NH_2), 1684 (CO). ^1H NMR (DMSO-d_6) δ: 3.67–3.71 (m, 4H, $2CH_2N$ morph), 3.36 (s, cryst. H_2O), 3.82–4.02 (m, 4H, $2CH_2O$ morph), 7.33–7.43 (m, 2H, H_7+H_8), 7.69 (t, $J = 6$, 1H, H_9), 7.98 (br s, 2H, NH_2, disappears with D_2O), 8.32 (d, $J = 6$, 1H, H_{10}). Anal. Calcd for $C_{15}H_{14}N_4O_3$. H_2O: C, 56.96; H, 5.10; N, 17.71. Found: C, 56.60; H, 4.88; N, 17.66.

5.1.13. Preparation of 4-hydroxy-2-methylthio-5*H*-[1]benzopyrano[4,3-*d*]pyrimidin-5-one 11. To a solution

of EtONa, obtained from Na (10 mmol, 0.23 g) and absolute ethanol (10 mL), was added *S*-methylisothiourea sulfate (5 mmol, 1.39 g). The mixture was stirred for 20 min, then Na_2SO_4 was filtered off and the solution was slowly added to methyl 4-chloro-2-oxo-2*H*-[1]benzopyrane-3-carboxylate **10** (5 mmol, 1.19 g) solved in absolute ethanol. The mixture was stirred at 60 °C for 3 h. The solid was filtered and recrystallized from DMF/H_2O (1:1).

White solid, mp: 260–261 °C, yield: 40%. IR (KBr) cm^{-1}: 3651 (OH), 3456 (OH), 1723 (C=O). ^1H NMR (DMSO-d_6) δ: 2.46 (s, 3H, SCH_3), 3.37 (m, OH+ cryst. H_2O, disappear with D_2O), 7.26–7.38 (m, 2H, H_7+H_8), 7.62 (t, $J = 6$, 1H, H_9), 8.26 (d, $J = 6$, 1H, H_{10}). Anal. Calcd for $C_{12}H_8N_2O_3S$. $^5/_2$ H_2O: C, 47.21; H, 4.29; N, 9.18. Found: C, 47.15; H, 3.90; N, 8.99.

5.1.14. Preparation of 4-chloro-2-methylthio-5*H*-[1]benzopyrano[4,3-*d*]pyrimidin-5-one 12. A mixture of 4-hydroxy-2-methylthio-5*H*-[1]benzopyrano[4,3-*d*]pyrimidin-5-one **11** (5 mmol, 1.30 g) and $POCl_3$ (50 mL) was heated at 150 °C for 5 h. The excess $POCl_3$ was evaporated under reduced pressure and the crude product was treated with ice. The white solid obtained was filtered and recrystallized from absolute ethanol/$CHCl_3$ (9:1).

White solid, mp: 230–231 °C; yield: 71%. IR ($CHCl_3$) cm^{-1}: 1744 (C=O). ^1H NMR ($CDCl_3$) δ: 2.73 (s, 3H, SCH_3), 7.26–7.46 (m, 2H, H_7+H_8), 7.69 (t, $J = 6$, 1H, H_9), 8.51 (d, $J = 6$, 1H, H_{10}). Anal. Calcd for $C_{12}H_7N_2ClO_2S$: C, 51.71; H, 2.53; N, 10.05. Found: C, 51.55; H, 2.54; N, 10.09.

5.1.15. General procedure for 2-methylthio-4-cycloamino-5*H*-[1]benzopyrano[4,3-*d*]pyrimidin-5-ones 4a–c. To a suspension of 4-chloro-2-methylthio-5*H*-[1]benzopyrano[4,3-*d*]pyrimidin-5-one **12** (5 mmol, 1.39 g) in anhyd toluene (50 mL) was added the suitable cycloamine (20 mmol) and the mixture was stirred at reflux for 3 h. After cooling, the organic solution was washed twice with water (20 mL), then dried ($MgSO_4$) and evaporated under reduced pressure. The crude oil was crystallized by addition of ethyl ether and the white solid obtained was filtered and recrystallized from absolute ethanol.

5.1.16. 2-Methylthio-4-pyrrolidino-5*H*-[1]benzopyrano-[4,3-*d*]pyrimidin-5-one 4a. Mp 174–176 °C; yield: 74%. IR (KBr) cm^{-1}: 1714 (CO). ^1H NMR ($CDCl_3$) δ: 1.82–2.12 (m, 4H, $2CH_2$ pyrr), 2.63 (s, 3H, SCH_3), 3.54 and 3.90 (2m, 4H, $2CH_2N$ pyrr), 7.20–7.38 (m, 2H, H_7+H_8), 7.58 (t, $J = 6$, 1H, H_9), 8.45 (d, $J = 6$, 1H, H_{10}). Anal. Calcd for $C_{16}H_{15}N_3O_2S$: C, 61.32; H, 4.82; N, 13.41. Found: C, 61.02; H, 5.00; N, 13.26.

5.1.17. 2-Methylthio-4-piperidino-5*H*-[1]benzopyrano-[4,3-*d*]pyrimidin-5-one 4b. Mp 160–161 °C; yield: 87%. IR (KBr) cm^{-1}: 1715 (CO). ^1H NMR ($CDCl_3$) δ: 1.71–1.78 (m, 6H, $3CH_2$ pip), 2.61 (s, 3H, SCH_3), 3.65–3.77 (m, 4H, $2CH_2N$ pip), 7.25–7.37 (m, 2H, H_7+H_8), 7.58 (t, $J = 6$, 1H, H_9), 8.44 (d, $J = 6$, 1H, H_{10}). Anal. Calcd for $C_{17}H_{17}N_3O_2S$: C, 62.37; H, 5.23; N, 12.83. Found: C, 62.60; H, 5.06; N, 12.89.

5.1.18. 2-Methylthio-4-morpholino-5*H*-[1]benzopyrano[4,3-*d*]pyrimidin-5-one 4c. Mp 175–176 °C; yield: 83%. IR (KBr) cm^{-1}: 1717 (CO). ^1H NMR ($CDCl_3$) δ: 2.61 (s, 3H, SCH_3), 3.73–3.82 (m, 4H, $2CH_2N$ morph), 3.84–3.90 (m, 4H, $2CH_2O$ morph), 7.26–7.40 (m, 2H, H_7+H_8), 7.62 (t, $J = 6$, 1H, H_9), 8.46 (d, $J = 6$, 1H, H_{10}). Anal. Calcd for $C_{16}H_{15}N_3O_3S$: C, 58.35; H, 4.59; N, 12.76. Found: C, 58.07; H, 4.66; N, 12.53.

5.1.19. General procedure for 2-methanesulfonyl-4-cycloamino-5*H*-[1]benzopyrano[4,3-*d*]pyrimidin-5-ones 5a–c. To an ice-cooled solution of the appropriate 2-methylthio derivative **4** (3 mmol) in $CHCl_3$ (20 mL), *m*-chloroperbenzoic acid (70–75%) (6 mmol, 1.5 g) was added in small portions. The mixture was stirred at room temperature for 12 h, diluted with $CHCl_3$ (25 mL) and washed once with $Na_2S_2O_5$ satd solution (25 mL), once with $NaHCO_3$ satd solution (25 mL) and once with water (25 mL). Finally, it was dried ($MgSO_4$) and evaporated under reduced pressure. Compounds **5a** and **5c** were purified by flash chromatography on silica gel with CH_2Cl_2 or $CHCl_3$/CH_3OH (9:1), respectively, as eluents. All the solids obtained were recrystallized from absolute ethanol.

5.1.20. 2-Methanesulfonyl-4-pyrrolidino-5*H*-[1]benzopyrano[4,3-*d*]pyrimidin-5-one 5a. Mp 218–219 °C; yield: 51%. IR (KBr) cm^{-1}: 1731 (CO), 1307, 1114 (SO_2). ^1H NMR ($CDCl_3$) δ: 1.96–2.17 (m, 4H, $2CH_2$ pyrr), 3.41 (s, 3H, CH_3), 3.55 (t, $J = 6.4$, 2H, CH_2N pyrr), 3.95 (t, $J = 6.4$, 2H, CH_2N pyrr), 7.32–7.46 (m, 2H, H_7+H_8), 7.70 (t, $J = 6$, 1H, H_9), 8.32 (d, $J = 6$, 1H, H_{10}). Anal. Calcd for $C_{16}H_{15}N_3O_4S$: C, 55.64; H, 4.38; N, 12.17. Found: C, 55.79; H, 4.40; N, 11.96.

5.1.21. 2-Methanesulfonyl-4-piperidino-5*H*-[1]benzopyrano[4,3-*d*]pyrimidin-5-one 5b. Mp 211–212 °C; yield: 83%. IR (KBr) cm^{-1}: 1727 (CO), 1313, 1135 (SO_2). ^1H NMR ($CDCl_3$) δ: 1.74–1.84 (m, 6H, $3CH_2$ pip), 3.40 (s, 3H, CH_3), 3.60–4.00 (m, 4H, $2CH_2N$ pip), 7.32–7.45 (m, 2H, H_7+H_8), 7.68 (t, $J = 6$, 1H, H_9), 8.50 (d, $J = 6$, 1H, H_{10}). Anal. Calcd for $C_{17}H_{17}N_3O_4S$: C, 56.81; H, 4.77; N, 11.69. Found: C, 56.74; H, 4.83; N, 11.52.

5.1.22. 2-Methanesulfonyl-4-morpholino-5*H*-[1]benzopyrano[4,3-*d*]pyrimidin-5-one 5c. Mp 228–229 °C; yield: 41%. IR (KBr) cm^{-1}: 1730 (CO), 1310, 1109 (SO_2). ^1H NMR ($CDCl_3$) δ: 3.41 (s, 3H, CH_3), 3.68–4.03 (m, 8H, $4CH_2$ morph), 7.28–7.48 (m, 2H, H_7+H_8), 7.70 (t, $J = 6$, 1H, H_9), 8.49 (d, $J = 6$, 1H, H_{10}). Anal. Calcd for $C_{16}H_{15}N_3O_5S$: C, 53.18; H, 4.18; N, 11.63. Found: C, 52.93; H, 4.24; N, 11.49.

5.1.23. General procedure for (2-methylthio-5*H*-[1]benzopyrano[4,3-*d*]pyrimidino)-4-cycloamines 6a–c. To a cooled mixture of the suitable 4-cycloamino-2-methylthio-5*H*-[1]benzopyrano[4,3-*d*]pyrimidin-5-ones **4a–c** (3 mmol) in anhyd. THF (12 mL), boron trifluoride etherate (12 mL), drop to drop, and sodium borohydride

(520 mg) in small portions were added. Then, diglyme (12 mL) was slowly added and the mixture was stirred at room temperature for 30 min and then refluxed for 3 h. After cooling, water (3 mL) and small portions of Na_2CO_3 until pH 6 were added. Aqueous solution was extracted twice with ethyl ether (20 mL), the organic phases were washed once with water (20 mL), dried ($MgSO_4$) and evaporated under reduced pressure. The crude oil obtained was treated with ethyl ether; the white solid obtained was filtered and recrystallized from absolute ethanol/$CHCl_3$ (9:1).

5.1.24. (2-Methylthio-5*H*-[1]benzopyrano[4,3-*d*]pyrimidino)-4-pyrrolidine 6a. Mp 163–165 °C; yield: 53%. ^{1}H NMR ($CDCl_3$) δ: 1.92–1.97 (m, 4H, $2CH_2$ pyrr), 2.58 (s, 3H, SCH_3), 3.57–3.72 (m, 4H, $2CH_2N$ pyrr), 5.34 (s, 2H, OCH_2), 6.93 (d, $J = 6$, 1H, H_7), 7.10 (t, $J = 6$, 1H, H_8), 7.34 (t, $J = 6$, 1H, H_9), 8.16 (d, $J = 6$, 1H, H_{10}). Anal. Calcd for $C_{16}H_{17}N_3OS$: C, 64.19; H, 5.72; N, 14.04. Found: C, 64.02; H, 5.73; N, 14.11.

5.1.25. (2-Methylthio-5*H*-[1]benzopyrano[4,3-*d*]pyrimidino)-4-piperidine 6b. Mp 129–130 °C; yield: 75%. ^{1}H NMR ($CDCl_3$) δ: 1.63–1.77 (m, 6H, $3CH_2$ pip), 2.59 (s, 3H, SCH_3), 3.23–3.38 (m, 4H, $2CH_2N$ pip), 5.06 (s, 2H, OCH_2), 6.95 (d, $J = 6$, 1H, H_7), 7.11 (t, $J = 6$, 1H, H_8), 7.37 (t, $J = 6$, 1H, H_9), 8.15 (d, $J = 6$, 1H, H_{10}). Anal. Calcd for $C_{17}H_{19}N_3OS$: C, 65.15; H, 6.11; N, 13.41. Found: C, 64.96; H, 6.08; N, 13.45.

5.1.26. (2-Methylthio-5*H*-[1]benzopyrano[4,3-*d*]pyrimidino)-4-morpholine 6c. Mp 155–156 °C; yield: 53%. ^{1}H NMR ($CDCl_3$) δ: 2.59 (s, 3H, SCH_3), 3.34 (t, $J = 5$, 4H, $2CH_2N$ morph), 3.82 (t, $J = 5$, 4H, $2CH_2O$ morph), 5.07 (s, 2H, OCH_2), 6.92 (d, $J = 6$, 1H, H_7), 7.10 (t, $J = 6$, 1H, H_8), 7.25 (t, $J = 6$, 1H, H_9), 8.12 (d, $J = 6$, 1H, H_{10}). Anal. Calcd for $C_{16}H_{17}N_3O_2S$: C, 60.93; H, 5.43; N, 13.32. Found: C, 60.78; H, 5.52; N, 13.32.

5.1.27. General procedure for (2-methanesulfonyl-5*H*-[1]benzopyrano[4,3-*d*]pyrimidino)-4-cycloamines 7a–c. To an ice-cooled solution of the suitable 2-methylthio-5*H*-[1]benzopyrano[4,3-*d*]pyrimidin-4-cycloamine **6** (3 mmol) in $CHCl_3$ (20 mL), *m*-chloroperbenzoic acid (70–75%) (6 mmol, 1.5 g) was added in small portions. The reaction mixture was stirred at room temperature for 12 h, diluted with $CHCl_3$ (25 mL) and washed once with $Na_2S_2O_5$ satd solution (25 mL), once with $NaHCO_3$ satd solution (25 mL) and once with water (25 mL). Finally, it was dried ($MgSO_4$) and evaporated under reduced pressure. The crude was purified by flash chromatography on silica gel with $CHCl_3$ as eluent. The white solid obtained was recrystallized from absolute ethanol/$CHCl_3$ (9:1).

5.1.28. (2-Methanesulfonyl-5*H*-[1]benzopyrano[4,3-*d*]pyrimidino)-4-pyrrolidine 7a. Mp 240–241 °C; yield: 56%. IR (KBr) cm^{-1}: 1306, 1113 (SO_2). ^{1}H NMR ($CDCl_3$) δ: 1.90–2.15 (m, 4H, $2CH_2$ pyrr), 3.36 (s, 3H, CH_3), 3.68–3.78 (m, 4H, $2CH_2N$ pyrr), 5.45 (s, 2H, OCH_2), 6.94 (d, $J = 8$, 1H, H_7), 7.12 (t, $J = 8$, 1H, H_8), 7.23–7.43 (m, 1H, H_9), 8.18 (d, $J = 8$, 1H, H_{10}). Anal. Calcd for $C_{16}H_{17}N_3O_3S$: C, 57.99; H, 5.17; N, 12.68. Found: C, 57.85; H, 4.91; N, 12.67.

5.1.29. (2-Methanesulfonyl-5*H*-[1]benzopyrano[4,3-d]pyrimidino)-4-piperidine 7b. Mp 202–203 °C; yield: 57%. IR (KBr) cm^{-1}: 1307, 1129 (SO_2). ^{1}H NMR ($CDCl_3$) δ: 1.60–1.80 (m, 6H, $3CH_2$ pip), 3.35 (s, 3H, CH_3), 3.38–3.45 (m, 4H, $2CH_2N$ pip), 5.13 (s, 2H, OCH_2), 6.97 (d, $J = 8$, 1H, H_7), 7.12 (t, $J = 8$, 1H, H_8), 7.40 (t, $J = 8$, 1H, H_9), 8.14 (d, $J = 8$, 1H, H_{10}). Anal. Calcd for $C_{17}H_{19}N_3O_3S$: C, 59.11; H, 5.54; N, 12.17. Found: C, 59.27; H, 5.41; N, 12.16.

5.1.30. (2-Methanesulfonyl-5*H*-[1]benzopyrano[4,3-*d*]pyrimidino)-4-morpholine 7c. Mp 219–220 °C; yield: 47%. IR (KBr) cm^{-1}: 1308, 1118 (SO_2). ^{1}H NMR ($CDCl_3$) δ: 3.38 (s, 3H, CH_3), 3.52 (t, $J = 4.4$, 4H, $2CH_2N$ morph), 3.85 (t, $J = 4.4$, 4H, $2CH_2O$ morph), 5.16 (s, 2H, OCH_2), 6.99 (d, $J = 8$, 1H, H_7), 7.14 (t, $J = 8$, 1H, H_8), 7.42 (t, $J = 8$, 1H, H_9), 8.14 (d, $J = 8$, 1H, H_{10}). Anal. Calcd for $C_{16}H_{17}N_3O_4S$: C, 55.32; H, 4.93; N, 12.10. Found: C, 55.20; H, 4.97; N, 12.09.

5.2. Pharmacology

5.2.1. Animals. Male Swiss mice (20–30 g), male Wistar rats (200–250 g) (Charles River) and male albino rabbits (3–3.5 kg) fed on a regular diet were used for the experiments. The animals were starved at 20 °C and fasted 18 h with free access to water before the experiments. All the experiments were performed according to ethical standard guidelines and were approved by Italian Ministry of Health (DL116/92).

The concentration-dependent inhibition of platelet aggregation and clot retraction were studied in vitro on rabbit or rat PRP by adding different concentrations (from 1 μM to 1 mM) of the test compounds. Acetylsalicylic acid (ASA), as well as the TXA2 antagonist SQ 29548, were used as reference drugs. The inhibitory potency of compounds, in both platelet aggregation and clot retraction tests, was expressed as IC_{50} (the concentration required to inhibit the response by 50% compared to the control experiment) and it was calculated by linear regression analysis of the specific concentration–response curves using the least-squares method.

To study the effects of the compounds on experimental thrombosis and bleeding time, the drugs or the vehicle was administered intraperitoneally at different doses (1–30 mg/kg/day) for 5 days in mice randomly distributed across treatments ($n = 10$–14 mice for each group). Animals' body weight, behaviour and physical appearance were monitored daily. The experiments were performed 1 h after the last pharmacological treatment. Acetyl salicylic acid (ASA) (50–100 mg/kg/day i.p.) and warfarin (1–3 mg/kg/day i.p.) were used as reference drugs. Dose–response curves of test compounds and reference drugs were constructed to evaluate antithrombotic and pro-haemorrhagic potency.

5.2.2. In vitro antiplatelet activity. Rabbit blood, anticoagulated with sodium citrate 3.8% 1 part citrate:9 part

blood, was obtained by cardiac puncture after CO_2 euthanasia and collected in plastic tubes.

After centrifugation for 15 min at 180 g to obtain platelet-rich plasma (PRP), the remaining blood was spun 10 min at 2000 g to obtain platelet poor plasma (PPP). Platelet aggregation was performed in the Aggrecorder PA 3220 (Menarini, Firenze) at 37 °C and in continuous stirring (1000 rpm), following Born's turbidimetric method.[32] Aggregation was recorded as the percent change in light transmission: the baseline was set using PRP and maximal transmission using PPP. PRP was preincubated at 37 °C for 5 min with solvent (dimethyl sulfoxide, at a maximal final concentration of 0.5%), the compounds under study or the reference drug at various concentrations before addition of platelet aggregant agents. Maximal platelet aggregation was induced with 5 μM ADP, 100 μM AA, or 2 μM U46619. Tests were performed within 3 h from blood collection to avoid platelet inactivation. The effects of test compounds were assessed as percent inhibition compared with the control sample. In blank tests, DMSO 0.5% did not interfere with platelet aggregation.

5.2.3. Clot retraction assay. Blood, collected through cardiac puncture from rats euthanized with CO_2, was centrifuged, as previously described, and properly diluted with Tyrode buffer (137 mM NaCl, 20 mM Hepes, 5.6 mM glucose, 1 mM $MgCl_2$, 2.7 mM KCl and 3.3 mM NaH_2PO_4, pH 7.4) to obtain PRP with about 200,000 platelets/μL. The assay was performed according to Davidson and Henry.[33]

Briefly, 450 μL aliquots of the above platelet suspension were added to siliconized glass tubes and incubated for 10 min with 5 μL solvent (DMSO 0.5% final concentration) or compounds under study at 37 °C. Fibrin clot retraction was induced by addition of 50 μL thrombin 20 U/mL. Clotting proceeded for 60 min at 37 °C. Clot formation and subsequent clot retraction were recorded visually by digital camera after 2 and 60 min from thrombin addition. Quantification of fibrin clot retraction was performed measuring by clot area by means of the NIH Image 1.67e software. Data were expressed as percentage of retraction = (area t_0 − area t)/area $t_0 \times 100$, where t_0 is the area of the clot 2 min after thrombin addition and t is the area at the test time (60 min).

5.2.4. Pulmonary thromboembolism. A modification of the method reported by DiMinno and Silver[30] was applied. Pulmonary acute thromboembolism was induced in mice by rapid intravenous injection in the tail vein of a mixture of 12 mg/kg collagen and 1 mg/kg epinephrine at doses selected to give a reproducible thromboembolism (about 70% of paralysis) in vehicle-treated mice. The loss of the righting reflex was considered as indication of acute paralysis. The number of paralysed animals was recorded up to 15 min from the injection of the thrombotic agents and the occurrence of paralysis within every group of animals was expressed as percentage of the treated mice.

5.2.5. Bleeding test. Bleeding test was determined, according to the method of Dejana et al.[31] Tails of lightly anaesthetized mice were transected at 2 mm from the tip and immersed in 1 mL of 37 °C saline for 2 min. Red blood cells were lysed by adding 20 μL triton 5% and absorbance of the solution was read at 560 nm (LKB, Ultraspec 4050). The amount of haemorrhage was estimated by linear regression analysis of a standard curve constructed from known volumes of mouse blood.

5.2.6. Statistical analysis. Data are expressed as means ± SEM. Differences between treated groups and vehicle-treated control group were analysed by χ^2 test for the evaluation of antithrombotic activity and by Mann–Whitney test for bleeding assay.

Otherwise stated unpaired Student's t test was applied to perform statistical analysis in the other studies with p value <0.05 or <0.01 being considered as statistically significant or highly significant, respectively.

5.2.7. Drugs. Drugs and reagents were obtained as follows: arachidonic acid. ADP, bovine thrombin (T3399), acetylsalicylic acid (ASA), calf collagen type III, epinephrine bitartrate, sodium citrate, dimethylsulpfoxide (DMSO) and Triton were from Sigma Chemical Co., St. Louis, MO. U46619 and SQ 29548 were from Caymanchem (MI, USA). ASA as lysine acetylsalicylate used for in vivo experiments was from Sanofi (Synthe Cabo). Drugs were dissolved in saline, except for collagen, which was suspended in isotonic glucose buffer according to Momo et al. (1992).[34] The compounds under study were dissolved in DMSO for in vitro experiments (0.5% DMSO final concentration) or suspended in saline for in vivo experiments.

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