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New Polycyclic Pyrimidine Derivatives with Antiplatelet In Vitro Activity: Synthesis and Pharmacological Screening

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Abstract—The preparation and the pharmacological screening of novel anti-aggregatory/antiphlogistic polycyclic pyrimidine derivatives are described. The compounds were developed starting from bioactive 2-aminobenzopyranopyrimidine derivatives in order to assess the importance of the benzopyrano[4,3-d]pyrimidine structure and the role of an amino basic moiety in position 2. Antiplatelet activity was assessed in vitro against ADP and arachidonic acid-induced aggregation in guinea-pig plasma. Anti-inflammatory/analgesic/antipyretic activities were studied in rat paw oedema, mouse writhing test and *E coli*-induced rat fever. Ulcerogenic and gastroprotective effects were also investigated in vivo on rat gastric mucosa. Among the tested compounds, the 5-substituted benzopyranopyrimidine derivatives **3d** and **4d** proved to be the most active antiplatelet agents as potent as acetylsalicylic acid against arachidonic acid-stimulated aggregation. Furthermore the 2-methylthio derivative **4d** was endowed with greater efficacy against ADP aggregation suggesting that additional non-TXA_2 dependent mechanisms are involved in its biological activity. Orally administered at 100 mg kg^{-1} in rats this latter compound displayed antiphlogistic acitivity comparable to indomethacin (10 mg kg^{-1}) coupled with an unusual gastroprotective effect on ethanol-induced ulcers. In conclusion, these findings indicate that the 5-pyrrolidino-2-methylthiobenzopyrano[4,3-d]pyrimidine **4d** fulfils the chemical requirements to exhibit antiplatelet activity associated with gastroprotective effect.

Introduction

It is well known that actually thromboembolic diseases are dramatic factors of morbidity and mortality. An effective way to prevent thrombotic events has been demonstrated to be the antiaggregant therapy with cyclo-oxygenase inhibitor aspirin (ASA).[1,2] However, a number of evidence indicates that this chronic antiplatelet therapy is frequently associated with gastro-intestinal adverse side effects.[3,4] Since multiple agonists can stimulate platelet aggregation[5] other antiplatelet agents acting with different mechanisms of action were developed in the recent past. However, a demand still remains for innovative antithrombotic drugs safer than those currently adopted.

For several years we are interested in synthetize new molecules as potential therapeutic agents in cardiovascular diseases: a number of 3,5-diphenyl-*1H*-pyrazole and of (3,5-diphenyl-*1H*-pyrazol-1-yl)pyrimidine derivatives, with antiplatelet and other significant activities, have been published.[6,7] In earlier experimental investigations[8,9] it has been demonstrated that several polycyclic fused molecules, sharing the benzopyran moiety, could present a platelet anti-aggregating activity in vitro superior or comparable to that of acetylsalicylic acid (ASA). More recently we have prepared and screened some 2-amino-*5H*-[1]benzopyrano[4,3-d]pyrimidin-5-amines **1a**–**f**[10] (Fig. 1) with the aim to examine carefully the relationships between the presence of basic moieties and antiphlogistic/antiplatelet activity. These compounds effective as analgesic/antipyretic agents exhibited a general inhibition of ADP-induced platelet aggregation in vitro, with **1d** and **1e** resulting to be slightly more active than ASA. Because compounds **1a**–**f** proved to be devoid of gastrolesive effects and also capable to prevent gastric mucosa from ethanol-induced ulcers in rats, we considered them interesting for a further investigation.

So we planned to perform different modifications of compounds **1a**–**f** in order to ascertain the importance of the benzopyrano[4,3-d]pyrimidine structure and the role

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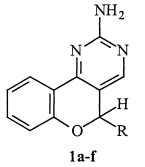
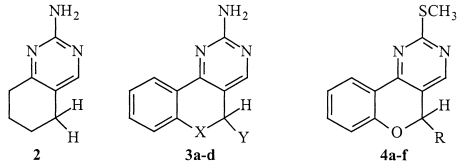


1a-f

Comp	R
1a	Isopropylamino
1b	t-Butylamino
1c	Cyclopropylamino
1d	Pyrrolidino
1e	Piperidino
1f	Morpholino

Figure 1.



2 3a-d 4a-f

3	X	Y
a	CH_2	H
b	S	H
c	O	H
d	O	C_6H_5

4	R
a	Isopropylamino
b	tert-Butylamino
c	Cyclopropylamino
d	Pyrrolidino
e	Piperidino
f	Morpholino

Figure 2.

of the basic moiety in position 2 of this heterocyclic system. According to this purpose we synthesized compounds **2** and **3a,b,c** (Fig. 2) maintaining an amino-substituted pyrimidine ring but fused with more simple or isoster moieties, while in the compound **3d** a phenyl group has been introduced in position 5. Furthermore, we prepared novel benzopyranopyrimidine derivatives **4a–f** (Fig. 2) in which the basic group in position 2 has been substituted with a methylthio function.

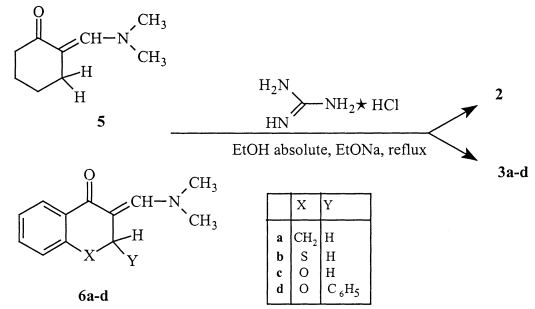
Thus, in the present work we evaluated the in vitro antiplatelet activity of these new derivatives in guinea-pig plasma aggregated by ADP and arachidonic acid (AA) in comparison with ASA, as well as their anti-inflammatory, analgesic and antipyretic activities. Additionally, for the most active compounds, we tested effects on gastric mucosa integrity in order to discuss the findings in comparison with the pharmacological data proven for the corresponding 2-amino derivatives (**1a–f**).

Chemistry

Compounds **2**[11] and **3a–d** were prepared by condensation of guanidine with the dimethylaminomethylene derivative of suitable ketones **5** or **6a–d** obtained by well known reactions[12–15] (Scheme 1). The preparation of dimethylaminomethylene flavanone **6d**[16] has been modified in order to increase the yield.

Compounds **4a–f** have been prepared following the previous synthetic method for compounds **1a–f** with the proper modifications (Scheme 2).

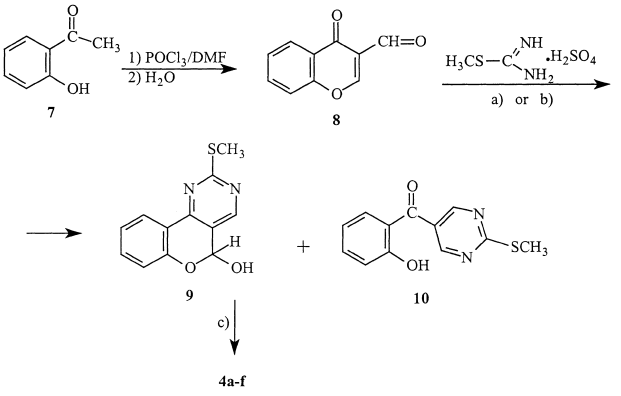
3-Formylchromone **8**, which was obtained from 2-hydroxyacetophenone 7 by a Vilsmeier reaction,[17] has been condensed with *S*-methylisothiourea sulfate to give the intermediate 5-hydroxy-2-methylthio-*5H*-[1]benzopyrano[4,3-d]pyrimidine **9**, probably according to the reaction pathway suggested by Ghosh in the case of the condensation of **8** with guanidine carbonate[18] (Scheme 3).



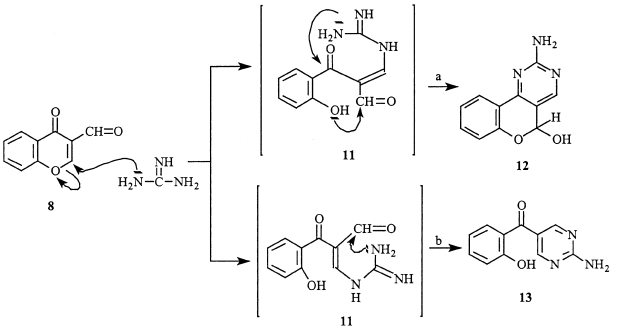
5 2 3a-d 6a-d

	X	Y
a	CH_2	H
b	S	H
c	O	H
d	O	C_6H_5

Scheme 1.



Scheme 2. (a) NaOH 1M, Et_3N, 80–90 °C; (b) EtOH absolute, C_2H_5ONa, reflux; (c) $TiCl_4$, toluene/anisole; primary/secondary amines.



Scheme 3.

In this case the reaction proceeds through a Michael addition at C-2 of the chromone moiety with concomitant opening of the pyrone ring; the intermediate **7** thus formed undergoes recyclization with subsequent water elimination according two different ways: route (a), which is energetically favoured, gives the predominant 2-amino-5-hydroxybenzopyrano[4,3-d]pyrimidine **12**, while route (b) leads to the by-product 2-amino-5-(2-hydroxybenzoyl)pyrimidine **13**. Ghosh and co-workers claimed that all the nucleophiles behave similarly with 3-formylchromone and their observations have also been supported by the subsequent study of Pene and Hubert-Habart.[19] Particularly, they demonstrated that in the presence of an alkali or a metal alkoxide (required to get the free bases) the yield of the by-product increases or, on the contrary, decreases when the formyl

group of **8** is opportunely masked by oxime, dioxolane or diacetyl groups.

The reaction of 3-formylchromone with *S*-methylisothiourea sulfate has already been carried out by Petersen[20] who obtained only the 5-hydroxy-2-methylthio-5*H*-[1]benzopyrano[4,3-d]pyrimidine **9** in the presence of 1M NaOH solution. We have now reinvestigated this reaction and we have verified that really compound **9** is the predominant product, in the Petersen method, but also a little amount of compound **10** has been formed. On the other hand, if the reaction is carried out in ethanol solution, in the presence of equimolar sodium ethoxide, the yield of **10** is greater.

Then, the hemiacetalic hydroxy-group in position 5 of the benzopyrano[4,3-d]pyrimidine system has been replaced with the proper amines in the presence of $TiCl_4$, according to a method already described (ref 10 and references cited therein).

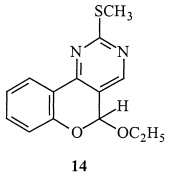
Finally, we observed that some of the compounds **4a**–**f**, transformed in hydrochlorides, can be converted into the corresponding 5-ethoxy-2-methylthio derivative **14** (Fig. 3) during the recrystallization process in absolute ethanol at 70–80 °C.

Preliminary studies suggested that this is a general behaviour for all the prepared compounds, but only as hydrochlorides. In fact no transformation has been evidenced during the recrystallization of the basic form of compounds **4**, or by refluxing them in absolute ethanol and sodium ethoxide. So, to understand if this is a common method to obtain a new series of 5-alkoxy derivatives, we are planning to study the influence of the temperature and the hydrochloric acid concentration on this reaction in the presence of different alcohols.

Results

In vitro antiplatelet activity

As regards the polycyclic pyrimidine derivatives **2**, **3a**–**d** a complete inhibition of ADP and AA evoked aggregation was detected only for compound **3b** (IC_{50} = 0.52 and 0.35 mM versus ADP and AA, respectively). In addition, only a partial inhibition of ADP induced aggregation was observed for compound **3c** (IC_{50} = 0.79 mM) while the phenyl substituted analogue **3d** caused an ASA-like antiplatelet effect against AA-aggregation (IC_{50} = 0.12 mM). As concerns the 2-methylthio substituted benzopyrano[4,3-d]pyrimidine derivatives three compounds (**4a**, **4c**, **4d**) out of six proved to be effective antiplatelet agents against ADP and AA induced aggregation of guinea pig PRP. In particular, the pyrrolidino derivative **4d** exhibited the highest activity being equipotent to ASA (IC_{50} = 0.061 mM) in inhibiting platelet aggregation elicited by AA (IC_{50} = 0.085 mM) and surpassing the reference drug when ADP-induced aggregation was considered. Indeed, while in this last model ASA produced only 40% inhibition, compound **4d** completely prevented the aggregation (IC_{50} = 0.18 mM). The isopropylamino substituted derivative **4a** as well as the cycloalkylamino **4c** displayed comparable antiplatelet activity against the two aggregation inducers employed. Finally, the remaining **4b**, **4e** and **4f** were able to prevent AA effect only at millimolar concentration and resulted ineffective against ADP evoked platelet activation even when tested at high concentrations (1 mM) (Table 1).



14

Figure 3.

In vivo experiments

In the carrageenan paw oedema assay an anti-inflammatory activity, comparable to the reference drug indomethacin (10 mg kg^{-1} os), was detected for benzothiopyranopyrimidine derivative **3b**, pyrrolidino **4d** and piperidino **4e** benzopyranopyrimidine derivatives (100 mg kg^{-1} os), which significantly inhibited (about 50%) rat paw swelling versus the control group. As regards the analgesic activity, no compounds exhibited a protective activity in writhing test. Among the molecules under study, 2-aminophenyl substituted derivative **3d** and cyclopropylamino derivative **4c** prevented the febrile response caused by *Escherichia coli* (Table 2).

As expected, the reference drug indomethacin (10 mg kg^{-1} os) caused gastric lesions while amongst the aforementioned effective compounds the pyrimidine derivatives **3b**, **4d** and **4e** (100 mg kg^{-1} os) did not produce

Table 1. In vitro antiplatelet activity, expressed as efficacy (% max effect) and potency (IC_{50} value), of acetylsalicylic acid (ASA) and pyrimidine derivatives under study on guinea-pig PRP[a] aggregation induced by ADP and arachidonic acid (AA)

Compounds	*n*	ADP-induced aggregation[b]		AA-induced aggregation[c]	
		Maximal inhibition (%)	IC_{50} (mM)	Maximal inhibition (%)	IC_{50} (mM)
2	6	[d]	—	56	0.91
3a	6	40	—	60	0.82
3b	6	100	0.52	100	0.35
3c	6	78	0.79	32	—
3d	6	52	0.82	100	0.12
4a	6	100	0.58	100	0.26
4b	6	[d]	—	100	0.58
4c	6	100	0.54	100	0.54
4d	6	100	0.18	100	0.08
4e	6	[d]	—	100	0.65
4f	6	[d]	—	100	0.75
ASA	10	43	—	100	0.06

[a]PRP, platelet-rich plasma.
[b]3 μM.
[c]50 μM.
[d]Ineffective up to 1 mM.

Table 2. Anti-inflammatory, analgesic and antipyretic activity (expressed as percentage of inhibition) of indomethacin and pyrimidine derivatives under study[a]

Compounds	Dose (mg kg^{-1} po)	n	Rat paw oedema	Mouse writhing test	Rat *E. coli* fever
2	100	10	11	27	0
3a	100	10	0	0	0
3b	100	10	44**	0	0
3c	100	10	0	0	0
3d	100	10	10	0	88*
4a	100	10	0	0	23
4b	100	10	0	0	33
4c	100	10	0	17	100**
4d	100	10	58**	29	7
4e	100	10	44**	0	6
4f	100	10	12	10	33
Indomethacin	10	28	45**	81**	93**

[a]Significance as compared to controls: * $p < 0.05$; ** $p < 0.01$

any mucosal erosions (Fig. 4a). Within this group of molecules, only derivatives **3b** and **4d**, at the same dosage, significantly protected gastric mucosa from ethanol-induced damage (Fig. 4b).

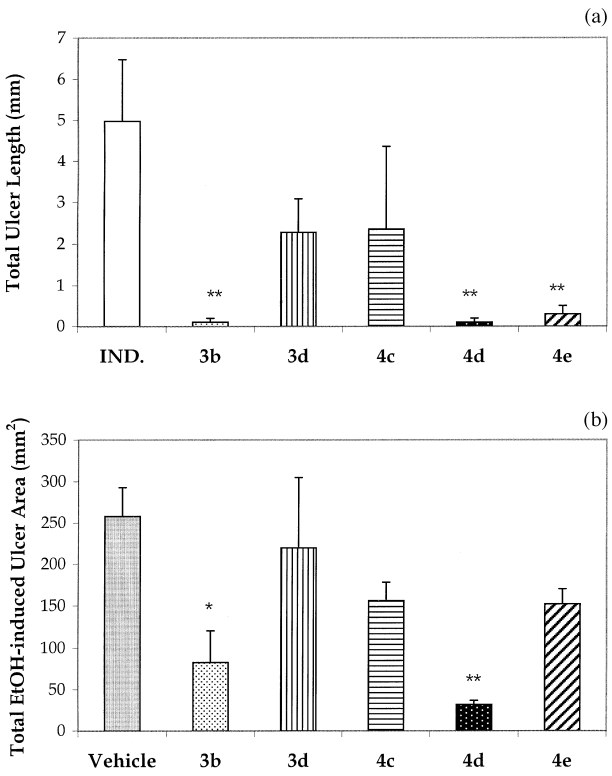


Figure 4. Effects on gastric mucosa following the oral administration of selected pyrimidine derivatives 100 mg kg^{-1} in conscious rats: (a) gastric ulcerogenic activity of the compounds under study and indomethacin (IND) (10 mg kg^{-1} os); (b) gastroprotective effects of the compounds and vehicle alone on ethanol-induced gastric ulcers. The total length of gastric lesions and the total area of ethanol-induced ulcers are considered respectively. Values represent the mean of 6–8 animals each and the vertical bars indicate SEM. ** $p < 0.01$; * $p < 0.05$.

Discussion

The different polycyclic pyrimidine derivatives **2**, **3a–d** generally displayed minor antiplatelet and analgesic/antipyretic activities with respect to the 2-aminobenzopyranopyrimidine derivatives previously studied **1a–f**. The sulfurated derivative **3b** coupled a moderate antiplatelet activity against ADP and AA-induced aggregation with a certain antiphlogistic action. It also was devoid of gastrolesive activity and significantly prevented the necrotizing activity of ethanol on rat gastric mucosa. Thus, these experimental data indicate that amongst the different manipulations performed at the polycyclic skeleton in compounds **2** and **3a–d** only the presence of S atom in the heterocyclic moiety **3b** did not negatively influence the in vivo and in vitro biological activity.

Within the remaining compounds under study, isopropylamino **4a**, cyclopropylamino **4c** and pyrrolidino **4d** derivatives revealed an attractive pharmacological profile. Unlike the conventional cyclooxygenase inhibitor ASA,[21,22] they potently prevented both AA and ADP stimulated aggregation of guinea-pig platelet in vitro. In detail, **4d** was as potent as ASA against AA and exhibited the highest activity in this experimental model being about 4 times more potent than **4a** and **4c**. Furthermore, since the three compounds displayed only 2–3-fold lower potency against ADP induced aggregation, it can be hypothesized that an additional, non TXA_2-dependent, mechanism can account for their anti-aggregating activity.

When orally administered in rats, **4c** and **4d**, **4e** acted as antipyretic and antiphlogistic agents, respectively. The compounds **4d** and **4e**, unlike indomethacin, did not cause any gastric mucosal damage and compound **4d** displayed a significant gastroprotective effect against ethanol-induced gastric ulcers. Since the occurrence of microvascular injury is involved in this ulcer model, it is likely that the anti-aggregating activity of such compounds can contribute to prevent the necrotic erosions induced by ethanol.[23]

By comparing the present data concerning compounds **4a–f** with the findings obtained for the **1a–f**[10] various considerations can be drawn. The presence of the 2-methylthio instead of the 2-amino group markedly impaired the in vivo biological activities, since within this new series only the cyclopropylamino **4c**, pyrrolidino **4d**, and piperidino **4e** derivatives partly maintained antiphlogistic/antipyretic properties while the corresponding aminoderivatives exhibited all these activities. As concerns the other isopropylamino **4a**, terbutylamino **4b** and morpholino **4f** derivatives a lack of any significant anti-inflammatory effect was reported. Furthermore, this replacement of the amino basic moiety in position 2 with a methylthio group seems to increase the in vitro antiplatelet activity. In particular, the 2-methylthio derivatives **4a**, **4c**, **4d** bearing respectively isopropylamino, cyclopropylamino and pyrrolidino substituents in position 5 of the heterocycle were more effective than the corresponding 2-amino analogues in inhibiting ADP-induced aggregation. They also prevented

the platelet response to AA with a good potency. Therefore, it seems reasonable to conclude that, within this series, the nature of the substituent introduced in position 5 could be crucial to determine the potential of the biological activities in vitro. We can infer that a lesser steric hindrance of isopropylamino, cyclopropylamino and pyrrolidino groups, in respect to the terbutylamino, piperidino or morpholino moieties, enhances antiplatelet activity. Furthermore, the pyrrolidino moiety of the most active compound **4d**, has unlike flexibility compared to that of cyclopropylamino or isopropylamino group. On the contrary, the basic strength of the 5-substituents does not seem important to raise the antiplatelet activity, because the pyrrolidino **4d** and piperidino **4e** derivatives have different level of activity in spite of their similar basicity. Moreover, it is noteworthy that among the new compounds only the pyrrolidino derivative **4d** prevented ethanol-induced gastric ulceration sharing the gastroprotective effect displayed by almost all the 2-amino derivatives. It follows therefrom that within the benzopyranopyrimidine series the occurrence of pyrrolidino moiety is associated with remarkable pharmacological properties apart from the presence of amino or methylthio 2-substitution.

In conclusion, from the present study it emerged that the potent antiplatelet and gastric sparing compound **4d** may be useful for the development of new drugs endowed with combined anti ADP/TXA_2 activity. The beneficial clinical effect of such antiaggregating agents can be speculated on the basis of the advantageous co-administration of antithrombotic drugs acting with different mechanisms in the treatment of vaso-occlusive disorders.[24,25]

Experimental

Materials and general methods

All chemicals were obtained from Sigma-Aldrich S.r.L. (Milan, Italy). Melting points are uncorrected and were measured with a Büchi 530 instrument (Büchi Laboratoriums-Technik AG, Flawil, Schweiz). IR spectra were recorded with a Perkin-Elmer 398 spectrophotometer (Perkin-Elmer, Milan, Italy). ^{1}H NMR were recorded on a Hitachi Perkin-Elmer R-600 (60 MHz) instrument (Perkin-Elmer, Milan, Italy); chemical shifts are reported as δ (ppm) relative to tetramethylsilane (TMS) as internal stardard; the signals were characterized as s (singlet), d (doublet), t (triplet), m (multiplet), br s (broad signal); J in Hz. Reactions were followed by TLC on Kieselgel $60F_{254}$ (DC-Alufolien, E. Merck, Darmstadt, Germany).

Analyses for C, H, N (±0.3% of the theoretical value), were determined with the Elemental Analyzer EA 1110 (Fison-Instruments, Milan, Italy).

General procedure for the preparation of 2-amino-5,6,7,8-tetrahydroquinazoline (2), 2-amino-5,6-dihydro-[e]quinazoline (3a), 2-amino-*5H*-[1]benzothiopyrano[4,3-d]pyrimidine (3b), 2-amino-*5H*-[1]benzopyrano[4,3-d]pyrimidine (3c) and 2-amino-5-phenyl-*5H*-[1]benzopyrano[4,3-d]pyrimidine (3d). Guanidine hydrochloride (1.9 g, 20 mmol) was added to a solution of sodium ethoxide obtained by dissolving sodium (0.46 g, 20 mmol) in dry ethanol (20 mL). After stirring at room temperature for 15 min. the solid obtained (NaCl) was removed by filtration and washed with dry ethanol (5 mL). The dimethylaminomethylene derivative of the suitable ketone (10 mmol) dissolved in dry ethanol (30 mL) was slowly added and the reaction mixture refluxed for 3–5 h. After concentration under reduced pressure the residue was poured into water (10 mL) and the suspension stirred for 1 h. The solid precipitated (white for **2**, **3a**, **c**, **d**, yellow for **3b**) was filtered, washed with water (10 mL) and ethyl ether (3 mL) and recrystallized by a proper solvent.

2. Yield: white crystals, 1.13 g, 75%. Mp = 211–212 °C (from ethanol) (lit: 212–213 °C[11]). Anal. calcd for $C_8H_{11}N_3$: C, 64.40; H, 7.43; N, 28.16. Found: C, 64.18; H, 7.42; N, 28.26.

3a. Yield: 76%. Mp = 187 °C (from ethanol:ethyl acetate, 1:2). ^{1}H NMR, δ ($CDCl_3$): 2.60–3.05 (m, 4H, $2H_5 + 2H_6$), 5.41 (br s, 2H, NH_2, disappear swith D_2O), 7.10–7.65 (m, 3H, $H_7 + H_8 + H_9$), 8.10–8.40 (m, 2H, $H_4 + H_{10}$). IR, cm^{-1} ($CHCl_3$): 3520–3410. Anal. calcd for $C_{12}H_{11}N_3$: C, 73.07; H, 5.62; N, 21.3. Found: C, 72.97; H, 5.63; N, 21.56.

3b. Yield: 74%. Mp = 167–168 °C (from ethanol:ethyl acetate, 1:1). ^{1}H NMR, δ ($CDCl_3$): 3.84 (s, 2H, $2H_5$), 5.55 (br s, 2H, NH_2, disappears with D_2O), 7.10–7.50 (m, 3H, $H_7 + H_8 + H_9$), 8.10–8.45 (m, 2H, $H_4 + H_{10}$). IR, cm^{-1} ($CHCl_3$): 3530–3420. Anal. calcd for $C_{11}H_9N_3S$: C, 61.37; H, 4.21; N, 19.52. Found: C, 61.17; H, 4.17; N, 19.52.

3c. Yield: 90%. Mp = 209 °C (from ethanol) (lit.: 209 °C[26]). ^{1}H NMR, δ ($CDCl_3$): 5.16 (s, 2H, $2H_5$), 6.74 (br s, 2H, NH_2, disappears with D_2O), 6.90–7.60 (m, 3H, $H_7 + H_8 + H_9$), 8.00–8.35 (m, 2H, $H_4 + H_{10}$). IR, cm^{-1} (KBr): 3330–3170.

3d. Yield: 80%. Mp = 164–165 °C (from ethanol). ^{1}H NMR, δ ($CDCl_3$): 5.37 (br s, 2H, NH_2, disappears with D_2O), 6.23 (s, 1H, H_5), 6.85–7.40 and 7.60–7.85 (2m, 3H, $H_7 + H_8 + H_9$), 7.47 (s, 5H, Ph), 8.10–8.30 (m, 2H, $H_{4+}H_{10}$). IR, cm^{-1} ($CHCl_3$):3540-3425. Anal. calcd for $C_{17}H_{13}N_3O$: C, 74.17; H, 4.76; N, 15.26. Found: C, 74.08; H, 4.74; N, 15.28.

2-(Dimethylaminomethylene)cyclohexanone **5**,[12] 2-(dimethylaminomethylene)-3,4-dihydro-1(*2H*)-naphtalenone **6a**,[13] 3-(dimethylaminomethylene)-4-thiochromanone **6b**,[14] 3-(dimethylaminomethylene)-4-chromanone **6c**,[15] were prepared following the literature references.

3-(Dimethylaminomethylene)-2-phenyl-4-chromanone **6d**[16] was prepared by the following modified method: Flavanone (3.5 g, 15 mmol) dissolved in DMFDMA (dimethylformamide dimethylacetal) (7 mL) was heated at 90–100 °C for 45 min. The excess DMFDMA was distilled under reduced pressure, ethyl ether (15 mL) was added to red oil residue and the pink-white solid crystallized was filtered. Yield: pink-white crystals. 1.75 g (42%);

Mp = 127–129 °C. Anal. calcd for $C_{18}H_{17}NO_2$: C, 77.40; H, 6.13; N, 5.01. Found: C, 77.41; H, 6.16; N, 4.97.

Preparation of 5-hydroxy-2-methylthio-*5H*-[1]benzopyrano[4,3-d]pyrimidine (9) and (2-hydroxyphenyl)-(2-methylthio-pyrimidin-5-yl)-methanone (10)

Method A. *S*-Methylisothiourea sulfate (1.39 g, 5 mmol) was added to a suspension of chromone-3-carbaldehyde **8** (1.74 g, 10 mmol) in 1 M NaOH (10 mL), followed by H_2O (20 mL) and triethylamine (0,5 mL). The reaction mixture was heated under stirring for 3 h at 70–80 °C. After cooling the 5-hydroxy-2-methylthio-*5H*-[1] benzopyrano[4,3-d] pyrimidine **9** precipitated as a rose-white solid which was filtered and recrystallized by ethanol (yield: 2.13 g, 87%).

The basic mother-waters were acidified to pH 5–6 with 1 M HCl and extracted twice with $CHCl_3$. The organic phases were dried ($MgSO_4$) and concentrated under reduced pressure to give a yellow oil which crystallized, with ether:petroleum ether (1:1) (5 mL), as a yellow solid corresponding to (2-hydroxyphenyl)-(2-methylthio-pyrimidin-5-yl)-methanone **10** (yield: 0.25 g, 10%).

Method B. *S*-methylisothiourea sulfate (1.39 g, 5 mmol) was added to a solution of sodium ethoxide obtained by dissolving sodium (0.23 g, 10 mmol) in dry ethanol; after stirring at room temperature for 10 min. the white solid obtained (Na_2SO_4) was removed by filtration and chromone-3-carbaldehyde 8 (1.74 g, 10 mmol) was added to the ethanolic solution. The mixture was refluxed for 6 h, then the solvent was evaporated under reduced pressure to give a orange-red solid as a mixture of compounds **9** and **10** (TLC: Kieselgel, $CHCl_3$:CH_3OH 9.5:0.5) The solid was dissolved in $CHCl_3$ and the organic phase was washed twice with 1 M NaOH (10 mL), dried ($MgSO_4$) and evaporated under reduced pressure to give a solid corresponding to the sole **9** which was recrystallized by ethanol (yield: 1.25 g, 51%). The basic solution was acidified (pH = 5–6) with 1 M HCl and extracted twice with $CHCl_3$ (10 mL). The organic phase was dried ($MgSO_4$) and evaporated under reduced pressure to give a yellow solid, recrystallized by ethanol, equivalent to the compound **10** (yield: 0.85 g, 35%).

9. Mp = 196–197 °C (lit: 196–198 °C[20]). ^{1}H NMR, δ (DMSO-d_6): 2.67 (s, 3H, SCH_3), 6.61 (s, 1H, H_5), 7.05–7.80 (m, 4H, $H_7 + H_8 + H_9$ + OH, 1H disappears with D_2O), 8.20–8.50 (m, 1H, H_{10}), 8.74 (s, 1H, H_4). IR, cm^{-1} ($CHCl_3$): 3000–3400 (OH).

10. Mp = 112–114 °C. ^{1}H NMR, δ ($CDCl_3$):2.67 (s, 3H, SCH_3), 6.80–7.30 and 7.45–7.85 (2m, 4H, Ar), 8.87 (s, 2H, pyrim.), 11,70 (s, 1H, OH, disappears with D_2O). IR, cm^{-1} ($CHCl_3$): 3460–2760 (OH); 1617 (C=O). Anal. calcd for $C_{12}H_{10}N_2O_2S$: C, 58.58; H, 4.09; N, 11.37. Found: C, 58.43; H, 3.95; N, 11.22.

General procedure for *N*-substituted 2-methylthio-*5H*-[1]benzopyrano[4,3-d]pyrimidin-5-amines (4a–f)

$TiCl_4$ (1.20 mL, 11 mmol) was added by stirring to an ice-cooled solution of dry toluene (40 mL) and anisole (2 mL). The freshly distilled relevant amine (5 mL) in dry toluene (5 mL) was then added and the coloured resulting mixture (orange with primary and green with secondary amines) was treated with **9** (2.15 g, 10 mmol) and a further excess of the same amine (3 mL) in dry toluene (10 mL).

After refluxing for 6 h, the orange-yellow suspension was cooled and then concentrated ammonia solution (3 mL), isopropanol (2 mL) and Kieselgur (2 g) were added in succession by stirring.

The resulting slurry was filtered and washed with toluene. The organic phase was washed once with little water, dried ($MgSO_4$), concentrated under reduced pressure and chromatographed on Florisil (100–200 mesh). Finally the solvent was evaporated under reduced pressure and the yellow oil so obtained was crystallized from absolute ethanol. Compound **4d** is an oil which do not crystallize, so the purification was achieved by distillation in vacuo.

Compounds **4d** and **4e** have been converted into their corresponding hydrochlorides with a saturated hydrogen chloride ethereal solution. When these salts have been heated for recrystallization in absolute ethanol at 70–80 °C both were converted into the same 5-ethoxy-2-methylthio-*5H*-[1]benzopyrano[4,3-d]pyrimidine **14**: white crystals, mp = 96–97 °C. ^{1}H NMR, δ ($CDCl_3$):1.20 (t, J = 6.6, 3H, CH_3), 2.66 (s, 3H, SCH_3), 3.85 (q, J = 6.6, 2H, CH_2), 6.17 (s, 1H, H_5), 7.00–7.70 (m, 3H, $H_7 + H_8 + H_9$), 8.20–8.55 (m, 2H, $H_{10} + H_4$). Anal. calcd for $C_{14}H_{14}N_2O_2S$: C, 61.29; H, 5.14; N, 10.21. Found: C, 61.12 ; H, 5.05; N, 10.42.

4a. Yield: 82%. Mp = 93 °C (from absolute ethanol). ^{1}H NMR, δ ($CDCl_3$): 0.90–1.35 (m, 6H, $2CH_3$), 2.13 (br s, 1H, NH, disappears with D_2O), 2.61 (s, 3H, S-CH_3), 3.10–3.70 (m, 1H, CH-isop.), 6.03 (br s, 1H, H_5, became s after exchange with D_2O), 6.90–7.70 and 8.10–8.50 (2m, 4H, Ar), 8.45 (s, 1H, H_4). Anal. calcd for $C_{15}H_{17}N_3OS$: C, 62.69; H, 5.96; N, 14.62. Found: C, 62.80; H, 5.88; N, 14.67.

4b. Yield: 81%. Mp = 117–118 °C (from absolute ethanol). ^{1}H NMR, δ ($CDCl_3$): 1.26 (s, 9H, $3CH_3$), 1.80–2.16 (br s, 1H, NH, disappears with D_2O), 2.63 (s, 3H, S-CH_3), 6.20 (br s, 1H, H_5 became s after exchange with D_2O), 6.85–7.56 and 8.10–8.50 (2m, 4H, Ar), 8.41 (s, 1H, H_4). Anal. calcd for $C_{16}H_{19}N_3OS$: C, 63.76; H, 6.35; N, 13.94. Found: C, 63.52; H, 6.30; N, 13.83.

4c. Yield: 69%. Mp = 104–105 °C (from absolute ethanol). ^{1}H NMR, δ ($CDCl_3$): 0.30–0.70 (m, 4H, $2CH_2$), 2.40–3.10 (m, 2H, CH-Cyclop. + NH, disappears with D_2O), 2.61 (s, 3H, S-CH_3), 5.96 (s, 1H, H_5), 6.90–7.60 and 8.15–8.48 (2m, 4H, Ar), 8.38 (s, 1H, H_4). Anal. calcd for $C_{15}H_{14}N_3OS$: C, 63.36; H, 4.96; N, 14.78. Found: C, 63.07; H, 5.24; N, 14.62.

4d. Yield: 69%. Bp = 190 °C/0.1 mmHg. ^{1}H NMR, δ ($CDCl_3$): 1.60–1.95 (m, 4H, $2CH_2$), 2.63 (s, 3H, S-CH_3), 2.60–3.20 (m, 4H, $2CH_2N$), 6.20 (s 1H, H_5), 6.83–7.60

and 8.10–8.45 (2m, 4H, Ar), 8.46 (s, 1H, H_4). Anal. calcd for $C_{16}H_{17}N_3OS$: C, 64.19; H, 5.72; N, 14.04. Found: C, 64.41 ; H, 5.68; N, 14.28.

4e. Yield: 89%. Mp = 97–98 °C (from absolute ethanol). ^{1}H NMR, δ ($CDCl_3$): 1.30–1.75 (m, 6H, $3CH_2$), 2.35–3.20 (m, 4H, $2CH_2N$), 2.62 (s, 3H, SCH_3), 5.98 (s, 1H, H_5), 6.80–7.60 and 8.10–8.45 (2m, 4H, Ar), 8.42 (s, 1H, H_4). Anal. calcd for $C_{17}H_{19}N_3OS$: C, 65.15; H, 6.11; N, 13.41. Found: C, 64.95; H, 6.13; N, 13.38.

4f. Yield: 87%. Mp = 117 °C (from absolute ethanol). ^{1}H NMR, δ ($CDCl_3$): 2.62 (s, 3H, SCH_3), 2.60–3.00 (m, 4H, $2CH_2N$), 3.5–3.8 (m, 4H, $2CH_2O$), 5.98 (s, 1H, H_5), 6.80–7.60 and 8.10–8.45 (2m, 4H, Ar), 8.45 (s, 1H, H_4). Anal. calcd for $C_{16}H_{17}N_3O_2S$: C, 60.93; H, 5.43; N, 13.32. Found: C, 60.91; H, 5.44; N, 13.25.

Pharmacological methods

All the new polycyclic pyrimidine derivatives were screened for their in vitro antiplatelet activity in guinea-pig platelet-rich plasma inducing the aggregation by ADP or AA. In vivo experiments were performed in order to evaluate their antiphlogistic, analgesic and antipyretic activities in rat paw oedema, mouse writhing test and rat *E. coli*-induced pyrexia, respectively. Selected compounds were tested for their erosive effects on gastric mucosa and their gastroprotective activity against ethanol-induced gastric ulcers in rats.

In vitro antiplatelet activity

Male guinea-pig (Morini, S.Polo-RE, Italy) blood obtained by cardiac puncture after CO_2 euthanasia was collected in plastic tubes containing sodium citrate (3.8% w/v; 9 parts blood: 1 part sodium citrate). The blood was centrifuged for 10 min at 200 *g* to obtain platelet rich plasma (PRP) and from the remaining blood, which was centrifuged for 10 min at 2000 *g* platelet poor plasma (PPP) was produced. Platelet aggregation was performed in an Aggrecorder PA 3220 aggregometer (A. Menarini, Firenze, Italy) following Born's turbidimetric method.[27] Aggregation was recorded as percent change in light transmission: the baseline was set by using PRP and full transmission (100%) was set by using PPP. PRP (250 μL) was preincubated at 37 °C for 5 min with solvent (dimethyl sulphoxide, DMSO), the compounds under study or the reference drug ASA (from 10^{-5} to 10^{-3} M) before the addition of the platelet aggregatory agent. PRP aggregation was induced by 3 μM ADP (25 μL) (Sigma) or by 50 μM AA (Menarini) and using concentrations sufficient to achieve maximum aggregation. Tests were performed within 3 h to avoid platelet inactivation. The effects of test compounds and acetylsalycilic acid, used as reference drug, were determined as percent inhibition calculated from the total aggregation in 5 min. Control samples received the same volume addition of DMSO at the final concentration of 0.5%. This concentration of solvent did not interfere with platelet assay.

In vivo experiments

Wistar female rats (250–300 g) and Swiss female mice (25–35 g) were used (Morini, S.Polo-RE, Italy). The test compounds were suspended in 0.5% methylcellulose and were orally administered at 100 mg kg^{-1} in 0.5 ml/100 g body weight to rats and at 0.15 ml/100 g body weight to mice 1 h before the application of phlogogen, algogen and pyretogen agent. Indomethacin 10 mg kg^{-1} os was used as reference drug in all the experimental tests while control animals received an equivalent volume of vehicle alone. Antiphlogistic, analgesic, antipyretic and gastric ulcerogenic/gastroprotective activities were evaluated following the experimental procedures already described.[10] Briefly, antiphlogistic activity was studied in rats by inducing paw oedema through subplantar injection of carrageenan, analgesic activity was evaluated in mice by acetic acid writhing test and antipyretic activity was determined in rats with *E. coli*-induced fever. The pharmacological activities were expressed as the percentage of inhibition calculated from the difference in the response between the treated and the control group at the time the maximum noxious effect occurred.

Selected compounds were studied to estimate their ulcerogenic/gastroprotective effects. In detail, 5 h after oral administration of these molecules the acute gastric ulcerogenic activity was determined by examining rat stomachs by means of a stereomicroscope connected to an image analyser system (Leitz, ASM 68K) and the total length (mm) of the gastric lesions was considered for each stomach. For the study of the gastroprotective activity the molecules were administered 1 h before the necrotizing agent ethanol (1 mL/rat 90% v/v). One hour after the oral administration of ethanol, the stomachs were microscopically analyzed and the surface of each haemorrhagic lesion was measured.

Statistical analysis

The results were expressed as mean±SEM and the means were compared using Student's t-test, p value <0.05 or <0.01 being considered as statistically significant or highly significant, respectively.

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