Pergamon

Bioorganic & Medicinal Chemistry 10 (2002) 2873–2882

BIOORGANIC & MEDICINAL CHEMISTRY

Pyridazines. Part XXIX: Synthesis and Platelet Aggregation Inhibition Activity of 5-Substituted-6-phenyl-3(2*H*)-pyridazinones. Novel Aspects of Their Biological Actions†

Eddy Sotelo,[a] Nuria Fraiz,[b] Matilde Yáñez,[b] Vicente Terrades,[a] Reyes Laguna,[b] Ernesto Cano[b] and Enrique Raviña[a,*]

[a]*Laboratorio de Química Farmacéutica, Departamento de Química Orgánica, Facultad de Farmacia, Universidad de Santiago de Compostela, 15782 Santiago de Compostela, Spain*

[b]*Departamento de Farmacología, Facultad de Farmacia, Universidad de Santiago de Compostela, 15782 Santiago de Compostela, Spain*

Received 26 February 2002; accepted 7 May 2002

Abstract—A series of 6-phenyl-3(2*H*)-pyridazinones with a diverse range of substituents in the 5-position have been prepared and evaluated in the search for new antiplatelet agents. A significant dependence of the substituent on the inhibitory effect has been observed. The pharmacological study of these compounds confirms that modification of the chemical group at position 5 of the 6-phenyl-3(2*H*)-pyridazinone system influences both variations in the antiplatelet activity and the mechanism of action.

Under physiological conditions, the adhesion of platelets to the subendothelium of damaged vessel walls and subsequent platelet aggregation are critical events in haemostasis.[2,3] Nevertheless, the uncontrolled deposition of platelets on the thrombogenic surface, such as ruptured atherosclerotic plaques, followed by formation of larger aggregates are the key steps in the development of various acute vasoocclusive diseases including unstable angina, myocardial infarction, transient ischemic attacks, stroke or reocclusion following thrombolytic therapy or angioplasty.[4]

Aspirin, the primary antiplatelet therapy in use today, has been shown to reduce the risk of arterial thrombosis in placebo-controlled clinical trials. Despite the proven efficacy of aspirin, there are reasons to believe that substantial improvements in antiplatelet therapy can be made. Among these is the fact that aspirin and the other currently available orally administered antiplatelet agents, such as ticlopidine and clopidogrel, are selective platelet inhibitors, and thus inhibit some but not all agonist-induced pathways of platelet activation and recruitment. Indeed, up to one third of patients respond in a limited manner to aspirin therapy, as determined by circulating platelet aggregate assay.[5]

Several of the reasons discussed above have driven the search for new compounds that act on a common feature of platelet aggregation induced by different agonists, such as the platelet fibrinogen receptor antagonists that have become a target in the search for more efficient antiplatelet drugs.[6] Despite extensive research in the area of $GP_{IIb/IIIa}$ inhibitors, only limited progress has been made in the search for orally active anti-thrombotic drugs and, recently, several bleeding complications have been reported during the use of these compounds in clinical trials.[7] Thus, new agents having a broad mechanism of action are of great interest not only for use as drugs but also because they are pharmacological tools that provide important information about platelet function.

In recent years, the 6-phenyl-3(2*H*)-pyridazinone system has aroused a great deal of attention due to its structural relationship to the 5-aryl-2(1*H*)-pyridones and, in particular, to milrinone and amrinone, the prototypes of a series of non-glycoside, *non*-catecholamine-based

†Part of this work was presented at the 7th International Symposium on the Chemistry and Pharmacology of Pyridazines, Santiago de Compostela, Spain, September, 2000. For the previous paper in this series, see ref 1.

*Corresponding author. Tel.: +34-981-594628; fax: +34-981-594912; e-mail: qofara@usc.es

0968-0896/02/$ - see front matter
PII: S0968-0896(02)00146-3

drugs that have mixed ionotropic/vasodilator activity. 3(2*H*)-Pyridazinones, 2(1*H*)-pyridones and related compounds also show antiplatelet activity,[8] the inhibition of the cyclic adenosine monophosphate (c-AMP) phosphodiesterase (PDE III in cardiac muscle and platelets) being assumed to be the primary source of these activities. However, the exact mechanism of their antiplatelet effect has not yet been completely elucidated and some differences in biological effects between milrinone and amrinone (Chart 1) have been reported.[9]

The intensive study of these compounds has allowed the establishment of a five-point pharmacophore model for selective PDE III inhibitors.[10] The following characteristics have a special relevance in terms of this model: a strong dipole (carbonyl group) in the azine system adjacent to an NH group, a small lipophilic group in the 5-position, a flat topography and a hydrogen bonding region at the end of the molecule. There are several characteristic 3(2*H*)-pyridazinones that conform to this model and these include imazodan, zardaverine, pimobendan and bemoradan (Chart 1).

As a continuation of our studies on the chemistry and pharmacology of 6-aryl-5-substituted-3(2*H*)-pyridazinones,[11–16] and as part of an ongoing project aimed at the synthesis of new and selective PDE III inhibitors to be used as antiplatelet agents, we recently prepared several 6-aryl-3(2*H*)-pyridazinones having the general structure shown in Chart 2. The results of these studies have confirmed the critical effect of the group in the 5-position and the hydrogen-bonding region at the end of the phenyl ring on the inhibition of platelet aggregation.[17]

The pharmacological evaluation of these compounds allows us to describe the antiplatelet activity of a range of 5-substituted-6-phenyl-3(2*H*)-pyridazinones that act through a *non*-cAMPC PDE III-based mechanism, since the tested compounds did not increase intracellular levels of cAMP.[18,21,22] On the basis of the experimental results, which show that this family of compounds inhibits platelet aggregation induced by diverse stimuli, it was suggested that they were disrupting a common event in the activation pathways leading to aggregation. As part of our detailed pharmacological study of these compounds we have recently reported[26] several new findings regarding the mechanism of action of the 6-phenyl- and 6-thienyl-5-hydroxymethyl-4,5-dihydro-3(2*H*)-pyridazinones, which seem to inhibit platelet activation due to their ability to suppress the passage of calcium through the cytoplasmic membrane.[19]

Prompted by these results we describe here the synthesis and new pharmacological studies of a series of 5-substituted-6-phenyl-3(2*H*)-pyridazinones. Our aim was to establish more detailed structure–activity relationship in this series and evaluate the modification of the pharmacological profile induced by the substituents in the 5-position of the 6-phenyl-3(2*H*)-pyridazinone system.

Chemistry

The chemistry used in the construction of the 6-phenyl-3(2*H*)-pyridazinone core and the subsequent elaboration to the target compounds is represented in Schemes 1–3. Some of the compounds studied here as antiplatelet agents have been previously described in papers published by our group.[16,18] 5-Oxygenated-3(2*H*)-pyridazinones were obtained from 5-hydroxymethyl-6-phenyl-4,5-dihydro-3(2*H*)-pyridazinone (**2**)[13] (Scheme 1). Selective aromatization of this compound using copper(II) chloride in acetonitrile gave alcohol **5** in 55% yield. Treatment of compound **2** with Jones' reagent led to carboxylic acid **4** in a troublesome procedure that involved the aromatization of the heterocycle and oxidation of the hydroxymethyl group. The low stability of compound **2** under acidic conditions allows the methyl derivative **3** (90%) to be obtained by refluxing **2** in hydrochloric acid. Oxidation or acetylation of alcohol **5** gave aldehyde **6**[18] (80%) or acetate **7**[18] (96%), respectively. Bromination of **5** using carbon tetrabromide and triphenylphosphine in methylene chloride afforded the synthetically useful bromomethyl derivative **8**,[16] which was the starting material in the preparation of the ethers **9**[16] (57%) and **10**[16] (67%) and the thioether **11** (85%) (Scheme 1).

Condensation of aldehyde **6** with hydroxylamine hydrochloride in pyridine gave oxime **12**,[16] which was efficiently converted to nitrile **13**[16] (88%) by dehydration employing trifluoroacetic acid (Scheme 2). Ester **15** was prepared by oxidation of aldehyde **6** using pyridinium dichromate in methanol. Subsequent ammonolysis of compound **15** afforded amide **16**[16] in high yield (90%).

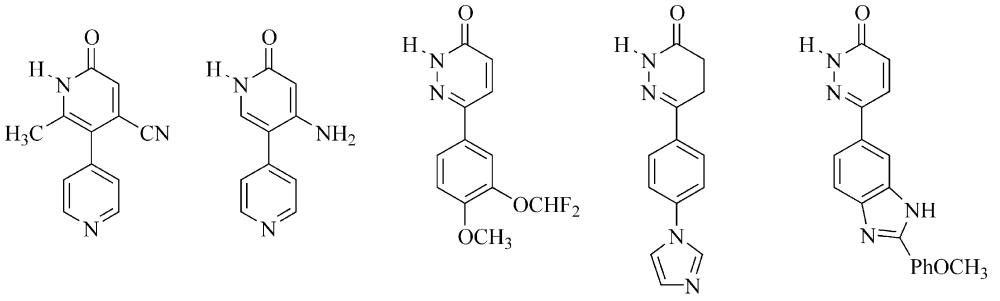


Chart 1.

Disappointingly, all our attempts to obtain compound **18** by direct addition of methyllithium to aldehyde **6** were unsuccessful (Scheme 2). We therefore prepared the methoxymethyl derivative **14** and reacted this with methyllithium to afford alcohol **17** in moderate yield (65%). Removal of the protecting group was accomplished by treatment of **17** with hydrochloric acid to give the desired alcohol **18** (50%). Finally, oxidation of **18** with manganese dioxide in tetrahydrofuran afforded methylketone **19** in high yield (89%). 6-Phenyl-5-halo-3(2*H*)-pyridazinones **20** and **21** were obtained by hydrazinolysis of the corresponding 2,3-dihalo-4-phenylcrotonolactones.[13]

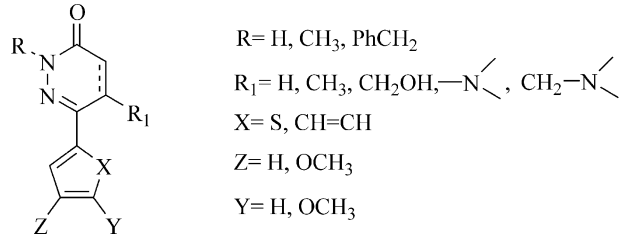
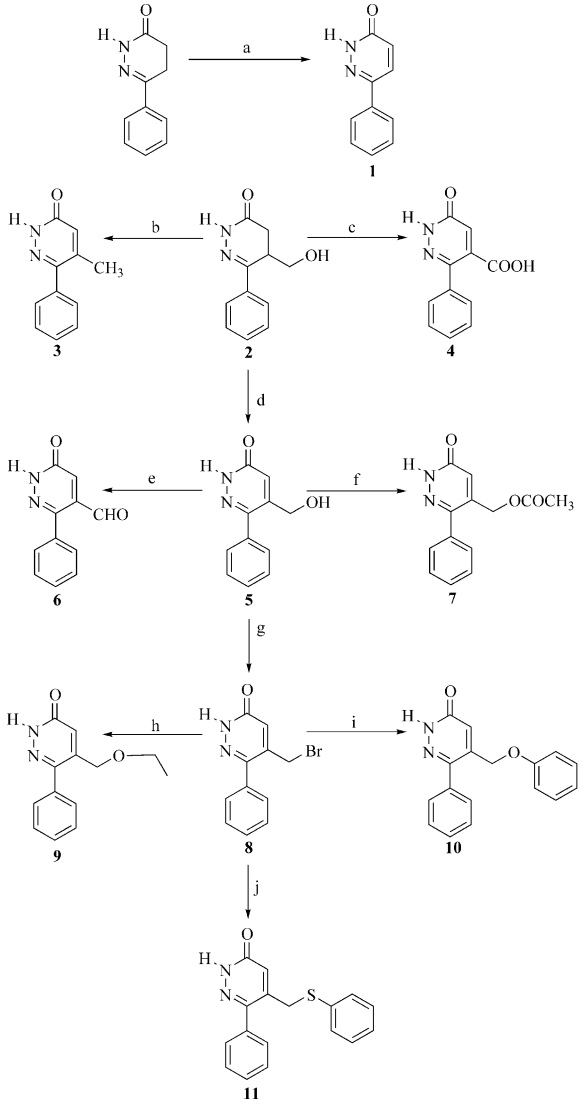
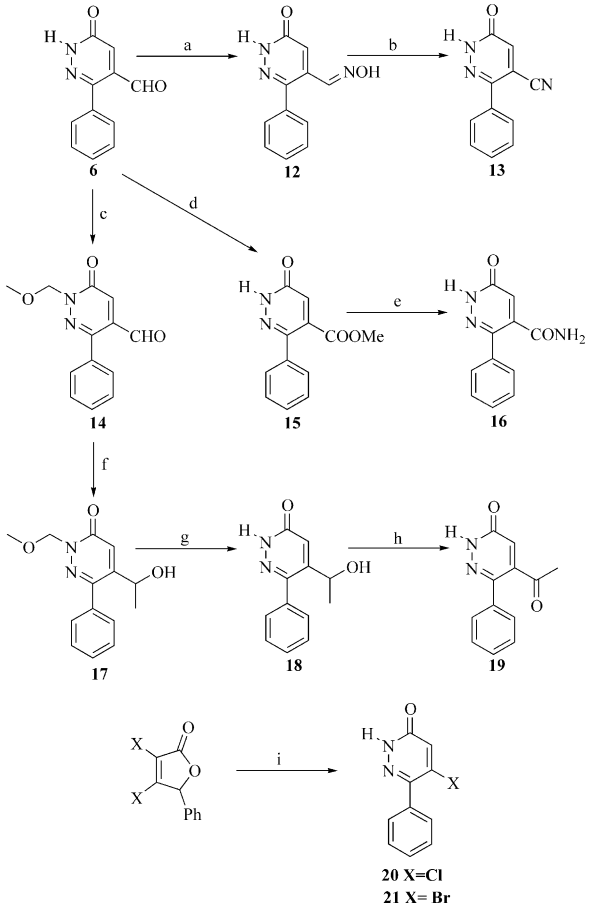


Chart 2.

5-Thio and 5-nitrogenated derivatives were prepared starting from 5-bromo-6-phenyl-3(2*H*)-pyridazinone (**21**) (Scheme 3). In contrast to our previous studies involving nitrogenated nucleophiles, compound **21** showed a high reactivity in the nucleophilic substitution reactions with mercaptans, to give the corresponding sulfur compounds in excellent yields. Thus, reaction of **21** with methylisothiourea in a mixture of methanol/ammonia afforded the methylthio derivative **25** in almost quantitative yield (96%), even in the presence of other nucleophiles such as the amidine or ammonia. Oxidation of the phenyl- or methylthio- derivatives **22** and **25**, using 3-chloroperbenzoic acid in methylene chloride, gave sulfones **26** and **33** in high yield. The ethers **27** and **28** and the phenylamine **30** were prepared in excellent yields by nucleophilic substitution of the methylsulfonyl group in **26**. Reaction of bromo derivative **21** with hydrazine hydrate or sodium azide gave compounds **23** and **30**, respectively. Direct amination of **21** using ammonium chloride/ammonia in a Parr reactor gave only moderate yields of the amine **31**,[16] and we therefore obtained this compound in an efficient manner by catalytic hydrogenation of azide **30** (Scheme 3).



Scheme 1. Reagents: (a) $CuCl_2$/acetonitrile; (b) 10% HCl; (c) CrO_3/H^+; (d) $CuCl_2$/acetonitrile; (e) MnO_2/THF; (f) $(CH_3CO)_2$/pyridine; (g) $CBr_4/PPh_3/CH_2Cl_2$; (h) EtONa/EtOH; (i) PhONa/EtOH; (j) PhSH/EtOH.



Scheme 2. Reagents: (a) $NH_2OH{\cdot}HCl$/pyridine; (b) CF_3COOH; (c) $ClCH_2OMe/DMAP/CH_2Cl_2$; (d) PDC/MeOH; (e) NH_3; (f): CH_3Li/THF −78 °C; (g) HCl 10%; (h) MnO_2/THF; (i) NH_2NH_2/EtOH.

The chemical structures of all compounds were confirmed by spectroscopic (IR, NMR, MS) and analytical data (see Experimental).

Results and Discussion

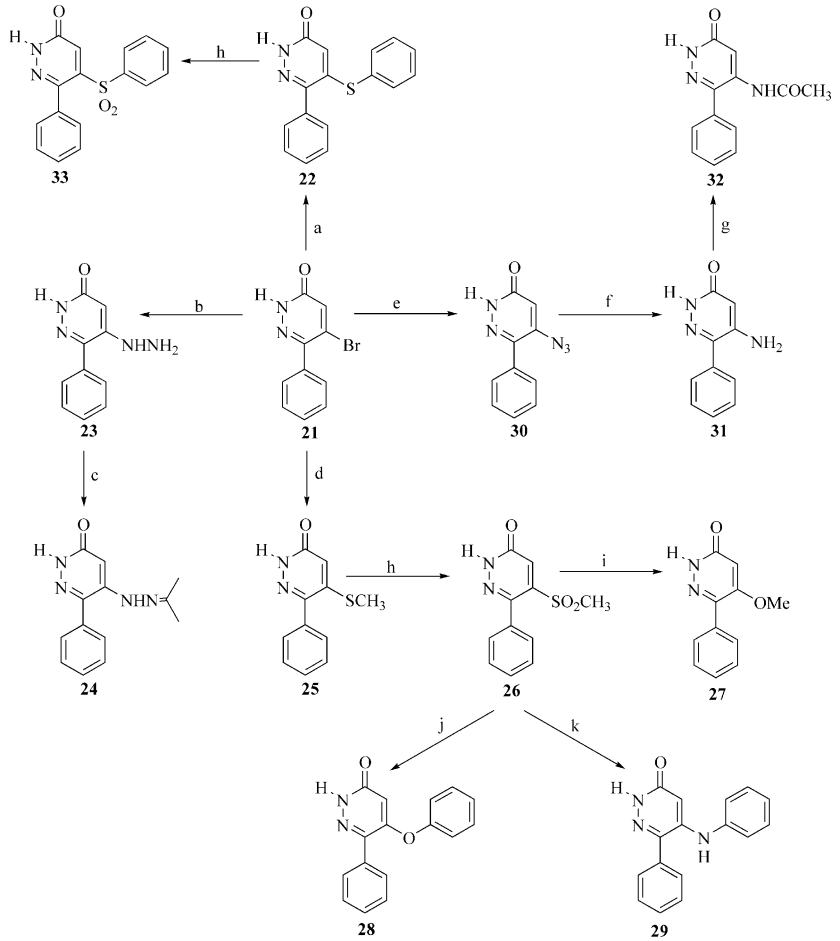
The platelet aggregation inhibitory activity of the 3(2*H*)-pyridazinone derivatives, obtained as described above, was examined on washed human platelets using thrombin as inducer of platelet aggregation. The results of these experiments are summarized in Table 1.

Many of the compounds studied inhibit platelet aggregation in a dose-dependent manner. Compound **22** shows the highest efficacy as a platelet aggregation inhibitor and has an IC_{50} value in the micromolar range (15 μM). Some of the pyridazinones **1**–**33** showed an interesting antiplatelet activity, although lower than the reference compound milrinone.

Several modifications of the oxygenated function in compound **5** reveal that, for example, oxidation to aldehyde **6** produces an increase in potency—in contrast, the acid **4** is inactive—whereas carboxylic acid derivatives **13** (X = CN), **14** (X = COOMe) and **16** ($X = CONH_2$) are only weakly actives. Conversion of **5** to the ether (**9** and **10**) or ester (**7**) produces a slight or marked increase in the antiplatelet activity, respectively.

A sharp increase in potency ($IC_{50} = 15\ \mu M$) was observed for compound **22** (X = SPh) but, in contrast, the homologue **11** ($X = CH_2SPh$) is completely inactive. If we consider the oxygenated analogues **10** ($X = CH_2OPh$) and **28** (X = OPh), it can be seen that the most potent compound is **10** while compound **28** induces platelet aggregation.

After considering the results indicated in Table 1, which illustrates the important effect that the chemical group in the 5-position of the 6-phenyl-3(2*H*)-pyridazinone system has on the antiplatelet activity, and in order to collect biological information important for ours SAR studies, several 3(2*H*)-pyridazinones were selected for further pharmacological evaluation and a detailed study of their mechanism of action. Initially, the anti-PDE III activity of several representative compounds (**6**, **7**, **10**, **13** and **22**) was determined in purified preparations obtained from vague of guinea pigs according to a previously reported protocol.[20] All compounds studied were found not to inhibit PDE III; thus, in agreement with our previous results,[19] these experiments confirm that the inhibition of the platelet function shown by 3(2*H*)-pyridazinones is not a c-AMP-based activity.



Scheme 3. Reagents: (a) $PhSH/K_2CO_3$; (b) NH_2NH_2; (c) CH_3COCH_3; (d) $(NH_2–C{=}NH)SCH_3/NH_3$; (e) NaN_3,/DMF; (f) H_2/Pd/AcOEt; (g) $(CH_3CO)_2O$/pyridine; (h) *m*-CPBA/CH_2Cl_2; (i) MeOH; (j) PhONa/THF; (k) $PhNH_2$/THF.

The subsequent pharmacological studies were conducted with the most active derivative in the series, that is compound **22**. This substance was found to inhibit platelet aggregation induced by different stimuli, including collagen and the calcium ionophore ionomycin (results not shown).

Stimuli that induce platelet aggregation differ fundamentally in the mechanism by which the process starts—generally by either the activation of a specific receptor or the massive entry of external calcium into the platelet (as occurs with ionomycin). Both possibilities have in common: an increase in the level of cytosolic calcium (with the associated initiation of processes that depend on the presence of this ion), the binding of fibrinogen to its receptor and the phosphorylation of certain proteins. These points lead us to believe that the mode of action of compound **22** centers on one of these key steps in platelet aggregation, a possibility that would certainly explain the effectiveness of this compound against the variety of stimuli employed.

The absence in compound **22** of the structural elements required to block the fibrinogen receptor[21–25] allows us to doubt any action in this regard. However, both an influence on the cytosolic calcium levels and on the phosphorylation state of the platelet proteins could be responsible for the anti-aggregatory effect observed for this compound and, for this reason, both possibilities were investigated.

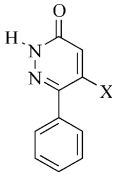
As far as the first effect is concerned, we have recently reported substances that are structurally related to compound **22** and are able to inhibit increases in cytosolic calcium levels, caused both by thrombin and ionomycin in human platelets.[19] It is therefore feasible that similar inhibiting properties could be associated with compound **22**. Related to second effect it should be considered that in cellular functions the phosphorylation of proteins has a different outcome depending on whether the phosphate group is incorporated in serine/threonine or tyrosine residues. For this reason, we next investigated the effects on protein phosphorylation.

The results obtained show that compound **22** plays a role in both processes outlined above and, as such, has the ability to inhibit increases in cytosolic calcium levels caused by thrombin as well as promoting an increase in the phosphotyrosine level of certain platelet proteins (changes in the level of phosphorylation of serine/threonine residues were not observed). Both effects were found to be dose-dependent (Figs 1 and 2).

Bearing in mind the fundamental role played by both phosphorylation processes and by the increase in cytosolic calcium levels in the development of the aggregation response, it is feasible that the action of compound **22** on one of these processes alone could explain the anti-aggregatory effect observed. In addition, if we consider the relationship between all the processes taking place in platelet activation, the effect of compound **22** on the phosphorylation may be the reason why cytosolic calcium levels do not increase or, conversely, the less marked increase in the calcium levels induced by the stimulus, due to inhibition by compound **22**, could explain the increase in protein phosphorylation. However, for the following reasons this situation does not seem to be probable:

1. The effect of compound **22** on the phosphorylation is more marked than its effect on the increase in cytosolic calcium, as evidenced by the fact that the increase in phosphorylation of proteins can be observed at concentrations where there is no effect on cytosolic calcium levels. Thus, doses lower than 5 μM caused a marked change on the tyrosine phosphorylation profile of several proteins whereas no changes on cytosolic calcium levels could be appreciated.
2. The anti-aggregatory effect shown by compound **22** appears to be more consistent with an inhibition of the increase in calcium levels than with the phosphorylation of proteins. This view is supported by the fact that concentrations at which the phosphorylation levels are modified, but calcium levels are not affected, do not lead to any noticeable anti-aggregatory effects. Indeed, doses lower than 5 μM did not induce platelet aggregation inhibition.
3. Other compounds from this and other related series, such as **2** and **5,** which have shown anti-aggregatory activity are also capable of inhibiting the rise in cytosolic calcium levels caused by various stimuli in platelets without modifying the phosphorylation profile (ref 19 and results not shown).

Table 1. Antiplatelet activity of the 5-substituted-6-phenyl-3(2*H*)-pyridazinones **1**–**33**



Compound	X	IC_{50} (mM)	Compound	X	IC_{50} (mM)
1	H	0.57	**19**	$COCH_3$	2.29
3	CH_3	1.20	**20**	Cl	—
4	COOH	—	**21**	Br	—
5	CH_2OH	1.50	**22**	SPh	0.015
6	CHO	0.31	**23**	$NHNH_2$	—
7	CH_2OCOCH_3	0.19	**24**	$NHN{=}C(CH_3)_2$	—
8	CH_2Br	—	**25**	SCH_3	—
9	CH_2OEt	0.67	**26**	SO_2CH_3	1.20
10	CH_2OPh	0.50	**27**	OMe	—
11	CH_2SPh	—	**28**	Oph	*
12	CH=NOH	*	**29**	NHPh	—
13	CN	1.00	**30**	N_3	0.90
15	COOMe	2.00	**31**	NH_2	—
16	$CONH_2$	1.00	**32**	$NHCOCH_3$	—
18	$CH(OH)CH_3$	—	**33**	SO_2Ph	0.70

— Inactive; * induces platelet aggregation.
Milrinone $IC_{50} = 0.0047$ mM.

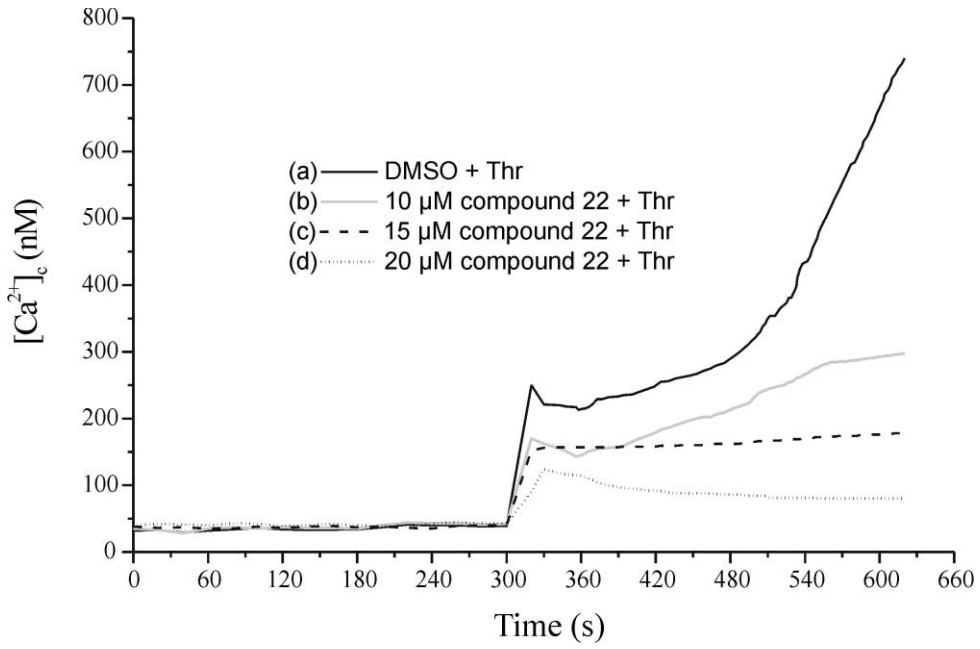


Figure 1. Effect of tested compound **22** on 0.25 U/mL thrombin-induced intracellular calcium increase in washed human platelets: (a) vehicle (DMSO); (b) 10 μM compound **22**; (c) 15 μM compound **22**; (d) 20 μM compound **22**.

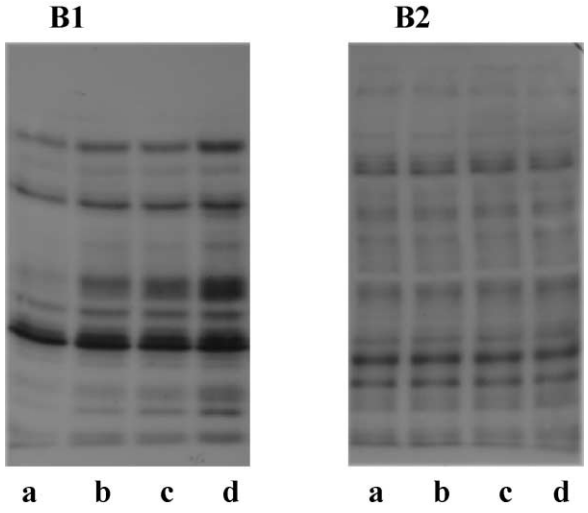


Figure 2. Anti-phosphotyrosine (**B1**) and anti-phosphoserine/threonine (**B2**) immunoblottings obtained from platelet lysates after SDS-PAGE and western blot, as described in Materials and Methods: (a) vehicle (DMSO); (b) 10 μM compound **22**; (c) 15 μM compound **22**; (d) 20 μM compound **22**.

For these reasons, the effects shown by compound **22** on protein tyrosine phosphorylation and on the increase in cytosolic calcium levels can be considered due to different cellular actions. In addition, the overall result is a synergy of the two effects on the basis that the anti-aggregatory activity of compound **22** is far greater than that of other analogues that affect only the calcium levels (e.g., **5**).

The phosphorylation state of any protein is the result of the balance between phosphorylation/dephosphorylation processes. Bearing this point in mind, the increase in the level of tyrosine phosphorylation, caused by compound **22**, can only be explained by an increase in the tyrosine-kinase activity or by an inhibition in the tyrosine phosphatase activity. In an attempt to understand the mechanism by which compound **22** acts, and in the absence of specific tests to elucidate the possible modes of action of this compound on the aforementioned enzymatic activities, we carried out a pharmacological study in which we evaluated its anti-aggregatory effect in the presence of an inhibitor of platelet tyrosine-kinase activity (tyrphostin B56, a representative member of the tyrphostin family). We found that neither the anti-aggregatory effect nor the increase in phosphorylation diminished in the presence of the inhibitor. For this reason, it is probable that compound **22** acts as a tyrosine-phosphatase inhibitor. This activity would partially explain the anti-aggregatory effect, because other compounds that are capable of promoting tyrosine-phosphorylation (by inhibiting the tyrosine-phosphatase activity) have also shown interesting properties as platelet aggregation inhibitors.[27,28]

In light of these results and other obtained with structurally-related compounds,[19] we can clearly conclude that the substituent in the 5-position of the 6-phenyl-3(2*H*)-pyridazinone system not only determines the antiplatelet activity of this series (quantitative aspect) but also the mechanism of action (qualitative aspect). This fact must be taken into account when SAR studies are undertaken with this type of compounds in order to avoid erroneous conclusions.

Experimental

Pharmacology

Preparation of washed platelets. Human platelet concentrates from blood anti-coagulated with citrate–phosphate–dextrose were obtained from the Centro de Transfusión de Galicia (Santiago de Compostela,

Spain). Platelets were purified by sedimentation through a discontinuous metrizamide gradient. For this purpose, 8 mL of platelet concentrate was layered onto a 10%/20% (1 mL/1 mL) metrizamide gradient and centrifuged at 1000*g* for 20 min. The resulting platelet band was recovered, diluted to 8 mL with washing buffer (NaCl, 140 mM; KCl, 5 mM; trisodium citrate, 12 mM; glucose, 10 mM; sucrose, 12.5 mM; pH 6) and centrifuged again at 1000 *g* for 20 min. Finally, the platelet band recovered from this step was resuspended in a modified Tyrode-HEPES buffer (HEPES, 10 mM; NaCl, 140 mM; KCl, 3 mM; MgCl, 0.5 mM; $NaHCO_3$, 5 mM; glucose, 10 mM; pH 7.4) affording a concentration of 3–3.5 10^{-8} platelets/mL. The calcium concentration in the extracellular medium was 2 mM.

Platelet aggregation studies. Platelet aggregation was measured using a dual channel aggregometer (Chronolog, Havertown, PA, USA). Each test compound was incubated with washed platelets at 37 °C for 5 min. Stimulus was then added to induce platelet aggregation, and the light transmission was monitored over a 5-min period. Platelet aggregation is expressed as the maximum change in light transmission during this period, with a 100% value being obtained when only stimulus, and not compound, was added.

Cytosolic free calcium measurements. Platelets recovered from the first washing step were incubated at 37 °C for 45 min with 3 μM fura-2 acetoxymethyl ester and then pelleted and resuspended as indicated above. Fura-2 fluorescence was monitored using a Shimadzu RF-5001 PC spectrofluorimeter. Excitation wavelengths were 340 and 380 nm and the emission wavelength was 485 nm. Cytosolic calcium concentration was calculated from the ratio of these two fluorescence intensities according to the method of Grynkiewicz et al.[29] with a dissociation constant (K_d) of 224 nM for Ca^{2+}.

Protein phosphorylation. After stimulation, human platelets were lysated with SDS/EDTA (2%/1 mM) in order to solubilize proteins and treated according to the Laemmli method.[30] The solubilized proteins were separated on SDS-polyacrylamide gel by electrophoresis (SDS-PAGE) using a 10% gel; known molecular weight proteins were used as standards. After SDS-PAGE, proteins were transferred to a PVDF membrane, which was then incubated for 2 h with appropriate monoclonal antibodies. In order to test selective phosphorylation sites (tyrosine or serine/threonine), each protein sample was run in two separate electrophoresis gels, which were transfered to PVDF membranes. One sample was incubated with 4G10 anti-phosphotyrosine antibody and the other with a mixture of anti-phosphoserine (1C8, 4A3, 4A9, 16B4) and anti-phosphothreonine (1E11, 4D11, 14B3) antibodies. Phosphoproteins were visualized by enhanced chemiluminiscence (ECL) using a goat anti-mouse antibody coupled to peroxidase.

Chemistry

Melting points were measured on a Gallenkamp melting point apparatus and are uncorrected. Infrared spectra (IR) were recorded on a Perkin-Elmer 1640 FTIR spectrophotometer. ^{1}H NMR spectra were obtained on Bruker WM250 and AM300 Hz spectrometers using tetramethylsilane as the internal standard (chemical shifts are in δ values, *J* in Hz). Mass spectra were determined on a Varian MAT-711 instrument. Elemental analyses were performed on a Perkin-Elmer 240B apparatus at the Microanalysis Service of the University of Santiago de Compostela. The progress of the reactions was monitored by thin layer chromatography with 2.5 mm Merck silica gel GF 254 strips, and the purified compounds each showed a single spot; unless otherwise stated iodine vapor and/or UV light were used for detection. Chromatographic separations were performed on silica gel columns by flash chromatography (Kieselgel 40, 0.040–0.063 mm).

6-Phenyl-3(2*H*)-pyridazinone (1). To a solution of the 4,5-dihydro-6-phenyl-3(2*H*)-pyridazinone (3.0 g, 16.3 mmol) in acetonitrile (25 mL) copper(II) chloride (4.38 g, 32 mmol) was added. The reaction mixture was refluxed during 50 min and then added to ice. The resulting solid was filtered off and recrystallized from isopropanol to give 2.81 g (95%) of **2**. Mp 203.0–205.0 °C, IR (KBr) cm^{-1}: 3950 (NH), 1647 (CO). ^{1}H NMR (dimethyl sulfoxide-d_6): δ 13.18 (s, 1H, NH), 8.00 (d, J = 9.9 Hz, 1H, CH), 7.84 (m, 2H, Ph), 7.46 (m, 3H, Ph), 6.98 (d, J = 9.9 Hz, 1H, CHCO). Anal. calcd for $C_{10}H_8N_2O$: C, 69.76; H, 4.68; N, 16.27. Found: C, 69.80; H, 4.70; N, 16.34.

5-Hydroxymethyl-6-phenyl-3(2*H*)-pyridazinone (5). To a solution of 4,5-dihydro-6-phenyl-3(2*H*)-pyridazinone (3.5 g, 17.1 mmol) in acetonitrile (25 mL) copper(II) chloride (4.61 g, 35 mmol) was added. The reaction mixture was refluxed during 35 min and then added to ice. The resulting residue was filtered off, purified by column chromatography (ethyl acetate/hexane 1:1) and then recrystallized from ethanol to provide alcohol **5** as colorless needles (1.9 g, 55%). Mp 198–201 °C, IR (KBr) cm^{-1}: 3200–2900 (NH), 1650 (CO pyridazinone), 1590 (C=C aromatics). ^{1}H NMR (dimethyl sulfoxide-d_6): 13.07 (s, 1H, NH, deuterium oxide exchangeable), 7.46–7.44 (m, 5H, aromatics), 6.93 (s, 1H, CHCO), 5.62 (s, 1H, OH), 4.26 (s, 2H, CH_2OH). Anal. calcd for $C_{11}H_{10}N_2O_2$: C, 65.34; H, 4.98; N, 13.85. Found: C, 65.38; H, 4.89; N, 13.85.

6-Phenyl-5-phenylthiomethyl-3(2*H*)-pyridazinone (11). To a solution of bromomethyl derivative **9** (0.15 g, 0.56 mmol) in ethanol (20 mL) mercaptobenzene (0.15 mL, 1.5 mmol) was added and the reaction mixture was refluxed during 1 h. The reaction mixture was concentrated under reduced pressure and the residue was purified by column chromatography (ethyl acetate/hexane 3:2) and then recrystallized from ethanol to provide **11** as colorless needles (0.14 g, 85%). Mp 171.9–173.1 °C, IR (KBr) cm^{-1}: 3050–2805 (NH), 1681 (CO), 1694 (aromatics). ^{1}H NMR (dimethyl sulfoxide-d_6): δ 13.13 (s, 1H, NH, deuterium oxide exchangeable), 7.51–7.44 (m, 5H, Ph), 7.39–7.18 (m, 5H, aromatics), 6.71 (s, 1H, CH–CO), 3.99 (s, 2H, CH_2–S). Anal. calcd for $C_{17}H_{14}N_2OS$: C, 69.36; H, 4.79; N, 9.52; S, 10.89. Found: C, 69.39; H, 4.81; N, 9.52; S, 10.90.

5-Formyl-2-methoxymethyl-6-phenyl-3(2*H*)-pyridazinone (14). To a mixture of aldehyde **6** (1.5 g 7.5 mmol), diisopropylethylamine (1.82 mL, 11.5 mmol) and a catalytic amount of 4-dimethylaminopyridine in anhydrous methylene chloride (25 mL) at 0 °C under an argon atmosphere methoxymethyl chloride (1.8 mL) was added slowly. The reaction mixture was stirred during 3 h and the solvent was concentrated under reduced pressure. The residue was poured into water and extracted with ethyl acetate. The organic layer was dried (Na_2SO_4) and the solvent removed under reduced pressure to give a solid, which was purified by column chromatography (ethyl acetate/hexane 1:1) to afford aldehyde **14** (1.65 g, 85%). Mp 79.3–81.0 °C, IR (KBr) cm^{-1}: 1710 (CHO), 1680 (CO), 1590 (aromatics). ^1H NMR (dimethyl sulfoxide-d_6): δ 9.82 (s, 1H, CHO), 7.55–7.47 (m, 5H, aromatics), 7.38 (s, 1H, H_4), 5.39 (s, 2H, $-CH_2$), 3.38 (s, 3H, CH_3). Anal. calcd for $C_{13}H_{12}N_2O_3$: C, 63.93; H, 4.95; N, 11.47. Found: C, 64.00; H, 4.96; N, 11.50.

5-Methoxycarbonyl-6-phenyl-3(2*H*)-pyridazinone (15). To a solution of aldehyde **6** (0.3 g, 1.5 mmol) in methanol (10 mL) pyridinium dichromate (0.58 g, 2 mmol) was added slowly and the mixture was stirred at room temperature during 6 h. The solution was poured into ice/water and the resulting solid was filtered off and recrystallized from methanol to give ester **10** (0.25 g, 70%). Mp 175.5–177.3 °C, IR (KBr) cm^{-1}: 3180–3050 (NH), 1650 (CO), 1730 ($COOCH_3$). ^1H NMR (dimethyl sulfoxide-d_6): δ 12.40 (s, 1H, NH), 7.89 (s, 1H, CH–CO), 7.50–7.39 (m, 5H, aromatics), 3.73 (s, 3H, OCH_3). Anal. calcd for $C_{12}H_{10}N_2O_3$: C, 62.61; H, 4.38; N, 12.17. Found: C, 62.65; H, 4.38; N, 12.21.

5-(1′-Hydroxyethyl)-2-methoxymethyl-6-phenyl-3(2*H*)-pyridazinone (17). To a solution of aldehyde **14** (0.5 g, 2.0 mmol) in dry tetrahydrofuran (25 mL) at −78 °C under an argon atmosphere was added slowly 1 M solution of methyllithium (2 mL). The reaction mixture was stirred for 3 h and then left at room temperature for 24 h. The reaction mixture was treated with water (25 mL) and the solution extracted with methylene chloride. The organic layer was dried (Na_2SO_4) and concentrated to give a colorless oil, which was purified by column chromatography (ethyl acetate/hexane 1:2) to afford a white solid (0.17 g, 40%) identified as **17**. Mp 117.3–119.0 °C, IR (KBr) cm^{-1}: 3235 (OH), 1680 (CO), 1580 (aromatics), 1150 (C–O–C). ^1H NMR (dimethyl sulfoxide-d_6): δ 7.53 (s, 5H, aromatics), 7.10 (s, 1H, H_4), 5.54 (m, 1H, CH), 5.38 (s, 2H, N–CH_2–O), 4.66 (s, 1H, OH), 3.41 (s, 3H, OCH_3), 1.03 (m, 3H, CH_3). Anal. calcd for $C_{14}H_{16}N_2O_3$: C, 64.20; H, 6.20; N, 10.76. Found: C, 64.24; H, 6.24; N, 10.78.

5-(1′-Hydroxyethyl)-6-phenyl-3(2*H*)-pyridazinone (18). To a solution of alcohol **17** (0.35 g, 1.3 mmol) in methanol (20 mL) 6 M hydrochloric acid (20 mL) was added and the mixture was stirred at 50 °C during 15 h. The reaction mixture was poured into ice and then extracted with ethyl acetate. The organic extracts were dried (Na_2SO_4) and concentrated under reduced pressure to give a solid, which was recrystallized from acetonitrile to afford **18** (0.20 g, 50%) as a white solid. Mp 186.0–188.0 °C, IR (KBr) cm^{-1}: 3255 (OH), 1680 (CO), 1590 (aromatics). ^1H NMR (dimethyl sulfoxide-d_6): δ 13.00 (s, 1H, NH, deuterium oxide exchangeable), 7.45 (m, 5H, aromatics), 6.95 (s, 1H, H_4), 5.43 (m, 1H, OH), 4.58 (m, 1H, CH), 1.01 (m, 3H, CH_3). Anal. calcd for $C_{12}H_{12}N_2O_2$: C, 66.65; H, 5.59; N, 12.96. Found: C, 66.69; H, 5.62; N, 12.98.

5-Acetyl-6-phenyl-3(2*H*)-pyridazinone (19). A mixture of alcohol **18** (0.15 g, 0.69 mmol) and manganese dioxide (0.60 g, 69 mmol) in tetrahydrofuran (30 mL) was stirred at room temperature during 48 h. The mixture was filtered through Celite and the solvent removed in vacuo to give ketone **29**, which was recrystallized from isopropanol (0.13 g, 89%). Mp 188.2–189.0 °C, IR (KBr): 3100–2900 (NH), 1702 ($COCH_3$), 1674 (CO). ^1H NMR (deuterochloroform): δ 12.64 (bs, 1H, NH, deuterium oxide exchangeable), 7. 44 (m, 5H, aromatics), 7.43 (s, 1H, H_4), 2.14 (s, 3H, CH_3). Anal. calcd for $C_{12}H_{10}N_2O_2$: C, 67.28; H, 4.71; N, 13.08. Found: C, 67.28; H, 4.70; N, 13.14.

5-Chloro-6-phenyl-3(2*H*)-pyridazinone (20). To a solution of 2,3-dichloro-4-phenylcrotonolactone (1.5 g, 6.6 mmol) in ethanol (10 mL) hydrazine hydrate (1.7 mL, 32 mmol) was added slowly and the mixture was heated under reflux during 3 h. The reaction mixture was allowed to cool and the solid that precipitated was filtered off and recrystallized from isopropanol (0.85 g, 63%). Mp 229.8–230.5 °C, IR (KBr): 3000–2900 (NH), 1680 (CO). ^1H NMR (dimethyl sulfoxide-d_6): δ 13.85 (bs, 1H, NH, deuterium oxide exchangeable), 7.58–7.50 (m, 5H, aromatics), 7.44 (s, 1H, H_4). Anal. calcd for $C_{10}H_7ClN_2O$: C, 58.13; H, 3.41; N, 13.56. Found: C, 58.18; H, 3.42; N, 13.58.

5-Bromo-6-phenyl-3(2*H*)-pyridazinone (21). To a solution of 2,3-dibromo-4-phenylcrotonolactone (1.5 g, 4.7 mmol) in ethanol (10 mL) hydrazine hydrate (1.2 mL, 23 mmol) was added slowly and the mixture was heated under reflux during 2 h. The reaction mixture was allowed to cool and the solid that precipitated was filtered off and recrystallized from isopropanol (0.71 g, 60%). Mp 231.0–232.0 °C, IR (KBr): 3000–2900 (NH), 1680 (CO). ^1H NMR (dimethyl sulfoxide-d_6): δ 13.78 (bs, 1H, NH, deuterium oxide exchangeable), 7.51 (m, 5H, aromatics), 7.45 (s, 1H, H_4). Anal. calcd for $C_{10}H_7BrN_2O$: C, 47.84; H, 2.81; N, 11.16. Found: C, 47.88; H, 2.83; N, 11.21.

6-Phenyl-5-phenylthio-3(2*H*)-pyridazinone (22). To a solution of bromo derivative **21** (0.5 g, 2.0 mmol) in ethanol (25 mL) potassium carbonate (0.15 g, 1.0 mmol) and mercaptobenzene (0.5 mL, 5.0 mmol) was added. The reaction mixture was heated under reflux during 6 h and the solvent was removed in vacuo. The resulting residue was added to ice to give a solid, which was filtered off and recrystallized from methanol (0.66 g, 88%). Mp 162.4–164.1 °C, IR (KBr): 3007 (NH), 1658 (CO). ^1H NMR (dimethyl sulfoxide-d_6): δ 13.08 (bs, 1H, NH, deuterium oxide exchangeable), 7.59–7.50 (m, 5H, aromatics), 7.39–7.25 (m, 5H, aromatics), 5.76 (s, 1H,

H_4). Anal. calcd for $C_{16}H_{12}N_2OS$: C, 68.55; H, 4.31; N, 9.99; S, 11.44. Found: C, 68.60; H, 4.32; N, 10.04; S, 11.47.

5-Hydrazino-6-phenyl-3(2*H*)-pyridazinone (23). A mixture of bromo derivative **21** (0.5 g, 2 mmol) and hydrazine hydrate (1 mL, 20 mmol) was heated under reflux during 5 h. The reaction mixture was allowed to cool and the solid that precipitated was filtered off and recrystallized from ethanol (0.71 g, 60%). Mp 194.0–195.2 °C, IR (KBr): 3305–2935 (NH, $NHNH_2$), 1624 (CO). ^{1}H NMR (dimethyl sulfoxide-d_6): δ 12.10 (bs, 1H, NH, deuterium oxide exchangeable), 7.44 (m, 5H, aromatics), 6.97 (s, 1H, H_4). Anal. calcd $C_{10}H_{10}N_4O$: C, 59.40; H, 4.98; N, 27.71. Found: C, 58.44; H, 5.02; N, 27.76.

5-Isopropylidenehydrazino-6-phenyl-3(2*H*)-pyridazinone (24). A solution of hydrazine **23** (0.25 g, 1.2 mmol) in acetone (10 mL) was heated under reflux during 2 h. The solvent was removed under reduced pressure to give a solid, which was recrystallized from methanol to afford a white solid (0.30 g, 95%) identified as **24**. Mp 247.7–248.2 °C, IR (KBr) cm^{-1}: 3025 (NH), 1680 (CO), 1590 (aromatics). ^{1}H NMR (dimethyl sulfoxide-d_6): δ 12.97 (s, 1H, NH, deuterium oxide exchangeable), 8.70 (s, 1H, NH), 7.80 (m, 2H, aromatics), 7.43 (m, 3H, aromatics), 7.09 (s, 1H, H_4), 2.04 (s, 3H, CH_3), 1.98 (s, 3H, CH_3). Anal. calcd for $C_{13}H_{14}N_4O$: C, 64.45; H, 5.82; N, 23.13. Found: C, 64.50; H, 5.82; N, 23.18.

5-Methylthio-6-phenyl-3(2*H*)-pyridazinone (25). To a suspension of bromo derivative **21** (0.50 g, 2.0 mmol) and *s*-methylisothiourea (0.60 g, 4.0 mmol) in ethanol (20 mL) aqueous ammonia (37%, 30 mL) was added. The reaction mixture was heated under reflux during 1 h. The solvent was removed under reduced pressure and the residue was poured into ice/water to give a solid, which was recrystallized from ethanol to afford **25** (0.41 g, 96%). Mp 244.3–246.0 °C, IR (KBr) cm^{-1}: 3440 (NH), 1666 (CO), 1580 (aromatics). ^{1}H NMR (dimethyl sulfoxide-d_6): δ 12.93 (s, 1H, NH, deuterium oxide exchangeable), 7.48 (m, 5H, Ph), 6.59 (s, 1H, CHCO), 2.39 (s, 3H, SCH_3). Anal. calcd for $C_{11}H_{10}N_2OS$: C, 60.53; H, 4.62; N, 12.83, S, 14.69. Found: C, 60.58; H, 4.61; N, 12.89; S, 14.74.

5-Methylsulfonyl-6-phenyl-3(2*H*)-pyridazinone (26). To a cooled (0 °C) solution of methylthio derivative **25** (0.25 g, 4.5 mmol) in methylene chloride (45 mL) *m*-chloroperbenzoic acid 77% (1.02 g, 8 mmol) was added slowly. The reaction mixture was stirred under these conditions for 1 h and then at room temperature for 12 h. The solvent was removed under reduced pressure and the residue was purified by column chromatography (ethyl acetate/hexane 3:1) and then recrystallized from isopropanol to give sulfone **26** (0.26 g, 90%). Mp 247.0–248.8 °C, IR (KBr) cm^{-1}: 3065–2925 (NH), 1695 (CO), 1575 (aromatics). ^{1}H NMR (dimethyl sulfoxide-d_6): δ 13.76 (s, 1H, NH, deuterium oxide exchangeable), 7.88 (s, 1H, CHCO), 7.55–7.49 (m, 5H, Ph), 2.93 (s, 3H, CH_3). Anal. calcd for $C_{11}H_{10}N_2O_3S$: C, 52.79; H, 4.03; N, 11.19; S, 12.81. Found: C, 52.81; H, 4.05; N, 11.24; S, 12.87.

5-Methoxy-6-phenyl-3(2*H*)-pyridazinone (27). A solution of sulfone **26** (1.05 g, 42 mmol) in methanol (25 mL) containing a catalytic amount of potassium carbonate was heated under reflux during 48 h. The solvent was removed under reduced pressure, the residue was purified by column chromatography (ethyl acetate/hexane 2:1) and then recrystallized from ethanol to give **27** (0.17 g, 40%). Mp 129.2–130.1 °C, IR (KBr) cm^{-1}: 2862 (NH), 1693 (CO), 1598 (aromatics). ^{1}H NMR (dimethyl sulfoxide-d_6): δ 12.82 (s, 1H, NH, deuterium oxide exchangeable), 7.59 (m, 2H, Ph), 7.14 (m, 3H, Ph), 6.31 (s, 1H, CHCO), 3.80 (s, 3H, OCH_3). Anal. calcd for $C_{11}H_{10}N_2O_2$: C, 65.34; H, 4.98; N, 13.85. Found: C, 65.34; H, 5.01; N, 13.87.

5-Phenoxy-6-phenyl-3(2*H*)-pyridazinone (28). To a solution of sodium phenoxide (0.14 g, 1.2 mmol) in tetrahydrofuran sulfone, **26** (0.30 g, 1.2 mmol) was added and the mixture was heated under reflux during 12 h. The solvent was removed under reduced pressure and the residue was poured into ice. The resulting solid was purified by recrystallization from ethanol to give **28** (0.26 g, 83%). Mp 202.8–204.1 °C, IR (KBr) cm^{-1}: 3500–3000 (NH), 1654 (CO), 1596 (aromatics). ^{1}H NMR (dimethyl sulfoxide-d_6): δ 13.05 (s, 1H, NH, deuterium oxide exchangeable), 7.73 (m, 2H, Ph), 7.54 (m, 6H, Ph), 7.31 (m, 2H, Ph), 6.92 (s, 1H, CHCO). Anal. calcd for $C_{16}H_{12}N_2O_2$: C, 72.72; H, 4.58; N, 10.60. Found: C, 72.88; H, 4.79; N, 10.56.

5-Phenylamino-6-phenyl-3(2*H*)-pyridazinone (29). A mixture of sulfone **27** (0.20 g, 0.8 mmol), 4-dimethylaminopyridine (50 mg) and aniline (0.14 g, 1.6 mmol) in tetrahydrofuran was heated under reflux during 8 h. The solvent was removed under reduced pressure and the residue was poured into ice. The resulting solid was purified by recrystallization from ethanol to give **29** (0.16 g, 78%). Mp 217.6–219.0 °C, IR (KBr) cm^{-1}: 3500–3200 (NH), 1669 (CO), 1578 (aromatics). ^{1}H NMR (dimethyl sulfoxide-d_6): δ 12.90 (s, 1H, NH, deuterium oxide exchangeable), 7.35 (m, 2H, Ph), 7.20 (m, 3H, Ph), 7.05 (m, 5H, Ph), 6.71 (s, 1H, CHCO), 3.63 (s, 1H, NH, deuterium oxide exchangeable). Anal. calcd for $C_{16}H_{13}N_3O$: C, 72.99; H, 4.98; N, 15.96. Found: C, 73.11; H, 5.07; N, 115.99.

5-Azido-6-phenyl-3(2*H*)-pyridazinone (30). A solution of **21** (1.05 g, 42 mmol) in DMF (15 mL) was treated with sodium azide (0.54 g, 82 mmol) and the mixture was stirred at room temperature during 24 h. The mixture was poured into ice/water and the resulting solid was filtered off and recrystallized from ethanol to give azide **30** (0.80 g). Mp 163.8–165.0 °C, IR (KBr) cm^{-1}: 2844 (NH), 2177 (N_3), 1692 (CO). ^{1}H NMR (dimethyl sulfoxide-d_6): δ 13.16 (s, 1H, NH, deuterium oxide exchangeable), 7.58 (m, 2H, Ph), 7.45 (m, 3H, Ph), 6.80 (s, 1H, CH–CO). Anal. calcd for $C_{10}H_7N_5O$: C, 56.34; H, 3.31; N, 32.85. Found: C, 56.39; H, 3.37; N, 33.01.

6-Phenyl-5-phenylsulfonyl-3(2*H*)-pyridazinone (33). To a cooled (0 °C) solution of phenylthio derivative **22** (0.35 g, 1.2 mmol) in methylene chloride (50 mL) *m*-chloroperbenzoic acid 77% (0.37 g, 2.5 mmol) was

added slowly. The reaction mixture was stirred under these conditions for 1 h and then at room temperature for 12 h. The solvent was removed under reduced pressure, the residue was purified by column chromatography (ethyl acetate/hexane 2:1) and then recrystallized from isopropanol to give sulfone **33** (0.31 g, 81%). Mp 172.0 °C (dec), IR (KBr) cm^{-1}: 3100–2850 (NH), 1683 (CO), 1576 (aromatics). ^1H NMR (dimethyl sulfoxide-d_6): δ 13.74 (s, 1H, NH, deuterium oxide exchangeable), 7.89 (s, 1H, CHCO), 7.70–7.25 (m, 10H, Ph). Anal. calcd for $C_{16}H_{12}N_2O_3S$: C, 61.53; H, 3.87; N, 8.97; S, 10.27. Found: C, 61.61; H, 3.96; N, 9.01; S, 10.34.

Acknowledgements

Financial support of this work by the Xunta de Galicia (Project XUGA 8151389) is gratefully acknowledged. We thank also to the Spanish *Instituto de Cooperación Iberoamericana* (ICI) for Eddy Sotelo's Doctoral Fellowship.

References and Notes

1. Sotelo, E.; Fraiz, N., Yañez, M., Laguna, R., Cano, E., Brea, J. M., Raviña, E. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 1575.
2. Leonard, E. F.; Turitto, V. T.; Vroman, L. (Eds.). In Blood in Contact with Natural and Artificial Surfaces. *Annals of the New York Academy of Sciences*, 1987; Vol. 516, pp 1–688.
3. Coller, B. S. *New Engl. J. Med.* **1990**, *322*, 33.
4. Falk, E. *Circulation* **1985**, *71*, 699.
5. Grotemeyer, K. H.; Scharafinski, H. W.; Husstedt, I. W. *Thromb. Res.* **1993**, *71*, 397.
6. Fujita, T.; Nasahide, N.; Inove, Y.; Shimohigushi, Y. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 1351. Liverton, N. J.; Armstrong, D. J.; Claremon, D.; Remy, C.; Baldwin, J. J.; Lynch, R. J.; Zhang, G.; Gould, R. J. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 483.
7. Iqbal, O.; Welanga, J. M.; Lewis, B. E.; Bakhos, M. *Drugs Today* **2000**, *36*, 8.
8. Thyes, M.; Lehman, H. D.; Gries, J.; Konig, H.; Kretsschmar, R.; Kunze, J.; Lebkucher, R.; Lenke, D. *J. Med. Chem.* **1983**, *26*, 800. Robertson, D. W.; Jones, N. D.; Krushinski, J. H.; Pollock, G. D.; Swartzendruber, J. K. *J. Med. Chem.* **1987**, *30*, 623.
9. Cody, V.; Wojtzack, A.; Davis, F. B.; Blas, S. *J. Med. Chem.* **1995**, *38*, 1990.
10. Moos, W. H.; Humbet, C. C.; Sircar, I.; Rithner, C.; Weishar, R. E.; Bristol, J. A.; McPhail, A. *J. Med. Chem.* **1987**, *30*, 1963, and references cited therein.
11. Raviña, E.; García-Mera, G.; Santana, L.; Orallo, F.; Calleja, J. M. *Eur. J. Med. Chem. Chim Ther.* **1985**, *20*, 475.
12. Terán, C.; Raviña, E.; Santana, L.; García-Domínguez, N.; García-Mera, G.; Fontela, J. A.; Orallo, F.; Calleja, J. M. *Arch. Pharm. (Weinheim)* **1989**, *322*, 331.
13. Raviña, E.; Terán, C.; Santana, L.; García-Domínguez, N.; Estévez, I. *Heterocycles* **1990**, *20*, 1967.
14. Laguna, R.; Montero, A.; Cano, E.; Raviña, E.; Sotelo, E.; Estévez, I. *Acta Pharm. Hungarica* **1996**, *66*, S43; *Chem. Abstr.* **1997**, *126*, 165993.
15. Estévez, I.; Raviña, E.; Sotelo, E. *J. Heterocyclic Chem.* **1998**, *35*, 1421.
16. Sotelo, E.; Raviña, E.; Estévez, I. *J. Heterocyclic Chem.* **1999**, *36*, 985.
17. Estévez, I. Doctoral Thesis, University of Santiago de Compostela, Spain, 1995.
18. Laguna, R.; Rodriguez-Liñares, B.; Cano, E.; Estévez, I.; Raviña, E.; Sotelo, E. *Chem. Pharm. Bull.* **1997**, *45*, 1151, and references cited therein.
19. Montero-Lastres, A.; Fraiz, N.; Laguna, R.; Cano, E.; Estévez, I.; Raviña, E. *Biol. Pharm. Bull.* **1999**, *22*, 1376.
20. Dal Piaz, V.; Giovannoni, M. P.; Castellana, C.; Palacios, J. M.; Beleta, J.; Doménech, T.; Segarra, V. *Eur. J. Med. Chem.* **1998**, *33*, 789.
21. Callahan, J. F.; Bean, J. W.; Burguess, J. L.; Eggleston, D. S.; Hwang, S. M.; Kopple, K. D.; Koster, P. F.; Nichols, A.; Peishoff, C. E.; Samanen, J. M.; Vasko, J. A.; Wong, A.; Huffman, W. F. *J. Med. Chem.* **1992**, *35*, 3970.
22. Callahan, J. F.; Newlander, K. A.; Burguess, J. L.; Eggleston, D. S.; Nichols, A.; Wong, A.; Huffman, W. F. *Tetrahedron* **1993**, *49*, 3479.
23. Ku, T. W.; Ali, F. E.; Barton, L. S.; Bean, J. W.; Bondinell, W. E.; Burguess, J. L.; Callahan, J. F.; Calvo, R. R.; Chen, L.; Eggleston, D. S.; Gleason, J. G.; Huffman, W. F.; Hwang, S. M.; Jakas, D. R.; Karash, C. B.; Keenan, R. M.; Kopple, K. D.; Miller, W. H.; Newlander, K. A.; Nichols, A.; Parker, M. F.; Peishoff, C. E.; Samanen, J. M.; Uzinskas, I.; Venslavsky, J. W. *J. Am. Chem. Soc.* **1993**, *115*, 8861.
24. Alig, L.; Edenhofer, A.; Hadvary, P.; Hurzeler, M.; Knopp, D.; Muller, M.; Steiner, B.; Trzeciak, A.; Weller, T. *J. Med. Chem.* **1992**, *35*, 4393.
25. Hartman, G. D.; Egberton, M. S.; Halczenko, W.; Laswell, W. L.; Duggan, M. E.; Smith, R. L.; Naylor, A. M.; Manno, P. D.; Lynch, R. J.; Zhang, G.; Chang, T. C. C.; Gould, R. J. *J. Med. Chem.* **1992**, *35*, 4640.
26. Dillon, A. M. R.; Heath, M. F. *Biochem. Biophys. Res. Commun.* **1995**, *212*, 595.
27. Bachelot, C.; Cano, E.; Grelac, F.; Saleun, S.; Druker, B. J.; Levy-Toledano, S.; Fischer, S.; Rendu, F. *Biochem. J.* **1992**, *284*, 923.
28. Villar, R.; Alvariño, M. T.; Flores, R. *Biochim. Biophys. Acta* **1997**, *1337*, 233.
29. Grynkiewicz, G.; Poenie, M.; Tsien, R. Y. *J. Biol. Chem.* **1985**, *260*, 3440.
30. Laemmli, U. K. *Nature (London)* **1970**, *227*, 680.