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Structure Activity Relationship of 4-Amino-2-thiopyrimidine Derivatives as Platelet Aggregation Inhibitors

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Abstract

Background: Platelet aggregation plays a pathogenic role in the development of arterial thrombi, which are responsible for common diseases caused by thrombotic arterial occlusion, such as myocardial infarction and stroke. Much efforts are directed toward developing platelet aggregation inhibitors that act through several mechanisms: the main antiplatelet family of COX-inhibitors, phosphodiesterase inhibitors, and thrombin inhibitors. Recently, the important role in the platelet aggregation of adenosine diphosphate (ADP)-activated $P2Y_{12}$ and $P2Y_1$ receptors, G-protein coupled receptors of the P2 purinergic family, has emerged, and their inhibitors are explored as potential therapeutic antithrombotics. $P2Y_{12}$ inhibitors, i.e. clopidogrel, prasugrel, ticagrelor, and cangrelor, are already used clinically to reduce coronary artery thrombosis risk and prevent acute coronary syndromes. The search for new $P2Y_{12}$ inhibitors, with better risk-to-benefit profiles is still ongoing.

Methods: Several years ago, our group prepared a series of 6-amino-2-thio-3H-pyrimidin-4-one derivatives that displayed an interesting platelet aggregation inhibiting activity. In order to probe the structure-activity relationships and improve their inhibitory effects of these compounds, we synthesized variously substituted 6-amino-2-thio-3H-pyrimidin-4-one derivatives and substituted 4-amino-2-thiopyrimidine-5-carboxylic acid analogues. All the synthesized compounds were

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

SUPPLEMENTARY MATERIAL

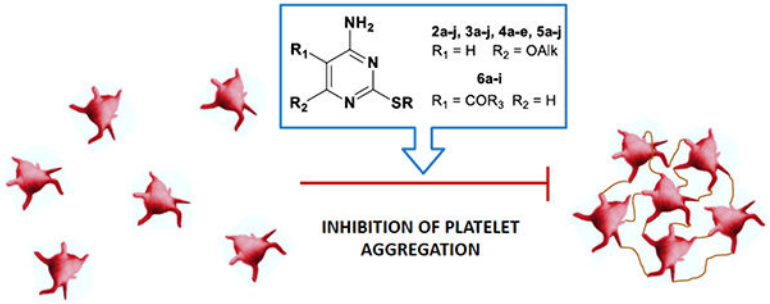
Supplementary material is available on the publisher's website along with the published article.

tested by light trasmission aggregometry (LTA) as inducers or inhibitors of platelet aggregation in citrated platelet-rich plasma (PRP).

Results: Among the 6-amino-2-thio-3H-pyrimidin-4-one derivatives, compounds **2c** and **2h** displayed marked inhibitory activity, with a capability to inhibit the ADP(10^{-6}M)-induced platelet aggregation by 91% and 87% at 10^{-4}M concentration, respectively. Selected 4-amino-2-thiopyrimidine-5-carboxylic acid derivatives were tested as $P2Y_{12}$ and $P2Y_1$ antagonists and found to display negligible activity.

Conclusion: These negative findings demonstrated that this heterocyclic nucleus is not a useful common pharmacophore for developing P2Y-dependent inhibitors of platelet aggregation. Nevertheless, compounds **2c** and **2h** could represent a new chemotype to further develop inhibitors of platelet aggregation.

Graphical Abstract



Keywords

Substituted 4-amino-2-thiopyrimidine; 6-amino-2-thio-3H-pyrimin-4-one; 4-amino-2-thiopyrimidine-5-carboxylic acid; synthesis; platelet aggregation inhibition

1. INTRODUCTION

Cardiovascular disorders are the most common cause of mortality in the developed world. The thrombotic diseases include myocardial infarction and cerebral stroke, acute coronary syndrome, angina, peripheral vascular disease, and thrombotic disorders such as atrial fibrillation. Usually, thrombotic diseases are caused by arterial occlusion by platelet-rich thrombi, which develop on diseased arteries [1–3].

Platelet-rich thrombi form when platelets aggregate to each other, as a consequence of complex activation mechanisms that are regulated by the interaction of platelet agonists with their specific platelet receptors. The antiplatelet drugs in therapeutic use belong to different classes, each one acting through a distinct mechanism, such as COX inhibitors [4], phosphodiesterase inhibitors [5], thrombin inhibitors [6], and $P2Y_{12}$ receptor antagonists, which have received a great attention in recent decades. The $P2Y_{12}$ and $P2Y_1$ receptors, both members of the P2 purinergic G protein-coupled receptors or metabotropic P2 receptors, play an important pathogenic role in arterial thrombosis [7–9]. They cooperate to mediate platelet aggregation induced by adenosine 5′-diphosphate (ADP); the $P2Y_1$ receptor induces the mobilization of ionized calcium from internal stores and mediates shape change and a

slight and rapidly reversible platelet aggregation, while the $P2Y_{12}$ receptor mediates a progressive and sustained aggregation not preceded by shape change. The selective tissue distribution of $P2Y_{12}$ makes it an attractive molecular target for therapeutic intervention [10].

Clopidogrel and prasugrel are members of the thienopyridine family, the first class of $P2Y_{12}$ receptor antagonists, which are currently used in clinical practice to reduce the risk of arterial thrombosis [11, 12]. Thienopyridines are prodrugs that need to be metabolized into their active metabolites, which irreversibly inhibit the $P2Y_{12}$ receptor [10]. Reversible drugs directly inhibiting the receptor have also been introduced: of these, ticagrelor is administered orally and cangrelor is administered intravenously [10].

Recently, several groups have reported $P2Y_{12}$ receptor antagonists belonging to various chemotypes, including piperazinyl glutamate-pyridines and pyrimidines [13], anthraquinones [14], phenylpyrazoles [15], and ethyl nicotinate derivatives such as AZD1283 [16], which was used to cocrystallize the receptor elucidating the interactions of the antagonist in the binding site [17]. These extensive efforts made by different research groups indicate a continuing interest in developing drug candidates for antiplatelet therapy (Fig. 1).

Previously, we reported a series of 6-amino-2-thio-3Hpyrimidin-4-one derivatives endowed with a weak inhibitory activity of platelet aggregation induced by ADP, supporting a possible $P2Y_{12}$ antagonism, as confirmed by $P2Y_{12}$ binding assays [18]. The large amount of data in the literature regarding $P2Y_{12}$ antagonist research, encouraged us to continue the development in this structural series as new, non-nucleosidic leads for $P2Y_{12}$ antagonists. As we reported, the toluene-4-sulfonic acid 6-amino-2-(2-aminoethylsulfonyl)-pyrimidin-4-yl ester (**1**) displayed the highest inhibitory activity and served as our reference lead compound for further investigation [18].

Here, we present a study to validate the substituted 6-amino-2-thio-3H-pyrimidin-4-one as a possible chemotype for the development of antiaggregatory agents, with a particular attention to evaluate the importance of different substitutions in platelet aggregation inhibition. In particular, we combined modifications at positions 2 and 4 of the 6-amino-2-thio-3H-pyrimidin-4-one scaffold with substituent groups that gave the best results in our previous series. Moreover, we eliminated the sulfonate group at position 4 in order to test its effect on activity. In addition, we prepared a small series of variously substituted 4-amino-2-thiopyrimidine-5-carboxylic acid derivatives directed toward investigating the effect of a different spatial organization of the substituents on the biological effects (Fig. 2).

2. CHEMISTRY

The substituted 6-amino-2-thio-3H-pyrimin-4-one derivatives (**2a-j, 3a-j, 4a-e**) were prepared as previously reported [18] (Scheme 1), starting from the 6-amino-2-thio-3H-pyrimin-4-one **7** through alkylation of the thio group under basic conditions in the presence of the appropriate alkyl halides (**8a-c**). The thio-substitued compounds **8a-c** were then treated with the corresponding alkyl- and arylalkyl halides in the presence of anhydrous

potassium carbonate to furnish the final compounds **2a-j, 3a-j, 4a-e**. The compounds **5a-j** were obtained through reaction of **2a-j** with tosyl chloride in pyridine. Although the ethyl-4-amino-2-thioprymidine-5-carboxylate **9** was commercially available, but too expensive, we synthesized it starting from ethyl-2-cyano-3-ethoxyacrylate and thiourea in the presence of sodium ethylate (freshly prepared), as reported in the literature [19].

The derivatives **6a-i** were obtained by alkylation of the thio group under the same basic conditions used for compound **7**, with various arylalkyl halides (Scheme 2). When the corresponding arylalkyl halides were not feasible to obtain commercially, they were prepared following standard conditions. As an example, the arylalkyl halide used to obtain compound **10b** was synthesized starting from 2-(4-hydroxyphenyl)acetic acid **13** which was protected at the carboxylic function as ethyl ester, alkylated at the hydroxy group with 4-chlorobutyl bromide to give ethyl 2-(4-(4-chlorobutoxy)phenyl)acetate. Finally, saponification of the ester and coupling to benzylamine furnished the desired *N*-benzyl-2-(4-(4-chlorobutoxy)phenyl)acetamide **14** (Scheme 3). The substituted compounds **10a-e, 10g-i** were treated with lithium hydroxide to give the corresponding carboxylic derivatives **6a,b, 11c-e, 11g-i**. **11c-e, 11g-i**, which were reacted with ethyl 3-aminobenzoate **15** in the presence of water-soluble carbodiimide (WSC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide) and 1-hydroxybenzotriazole (HOBt) under standard conditions to furnish **12c-e, 12g-i**, followed by saponification of the esters to afford the final compounds **6c-e, 6g-i**. The final compound **6f** was obtained through de-protection of the amino group of compound **6e** with trifluoroacetic acid at room temperature (Scheme 2).

3. RESULTS AND DISCUSSION

All the synthesized compounds were tested for their ability to induce platelet aggregation or to antagonize ADP-induced platelet aggregation, measured in citrated platelet-rich plasma (PRP) by light transmission aggregometry (LTA). The compounds were tested at a concentration of 10^{-4} M.

None of the tested compounds induced platelet aggregation. In contrast, all of them inhibited platelet aggregation induced by 10^{-6} M ADP. Compounds **2a, 2c, 2d, 2g** and **2h** displayed the highest inhibitory activity, as they highly inhibited platelet aggregation. However, the degree of inhibition of platelet aggregation induced by higher concentration of ADP (10^{-5} M) was very low or negligible with all tested compounds.

In the series derived from compound **7**, we introduced alkyl chains bearing hydrophilic groups in order to better understand the importance of hydrogen bonds on the 2 position thio function in the series **2a-j** and **3a-j**. We incorporated the 2-chlorobenzyl group that is present in the clopidogrel structure into compounds **4a-e**, and a *N*-ethyl-4-methylbenzenesulfonamide function, which displayed good results in our previous work, into compounds **5a-j**[18]. These substitutions were combined with different ether groups at the hydroxyl function on pyrimidine nucleus. Among these compounds, the series bearing the 2-aminoethyl group at 2-position (**2a-j**) displayed the highest potency in aggregation inhibition. Compound **2c** inhibited platelet aggregation induced by 10^{-6} M ADP by 91%, while the remaining compounds of this group displayed lower inhibitory efficacy. The

analogues **3a**, **3c**, **3d**, **3g** and **3h**, bearing a 2-hydroxyethyl group at the 2-position, were almost completely inactive, and the amino group in the side chain seemed important. Even the related compounds in the series bearing a *N*-ethyl-4-methylbenzenesulfonamide on the thio group at 2-position (**5a**, **5c**, **5d**, **5g** and **5h**), or the series **4a-e**, bearing a 2-chlorobenzyl substituent at the same position, did not show relevant inhibitory activity; this could imply that a steric hindrance around the thio function is not allowed (Table 3).

Considering the series **2a-j**, the influence on the activity of the 4-position substituents is unclear. First of all, the presence of a sulfonic group in this position is not as important as concluded in our previous study, where compound **1** was considered as a lead compound [18]. Moreover, the position of the substituents on the phenyl ring in the side chain seems to be more important than their chemical nature. As an example, the most active compounds of the series **2c** and **2h**, which bear electronically different methyl and chloro groups at the phenyl ring ortho position, displayed similar activities (9% and 13% inhibition respectively). This pattern is confirmed by the two analogues **2d** and **2g** (27% and 21% inhibition respectively), which bear methyl and chloro substituents, both at meta positions of the phenyl ring. Whereas, compounds **2c** and **2d** bearing the same methyl group as substituent on the phenyl ring, but at different positions (ortho and meta, respectively), displayed quite different inhibitory activities (9% and 27%). These findings could demonstrate that steric parameters are more relevant than electronic characteristics.

Similarly, in the series of compounds **5a-j**, compound **5c** displayed greater inhibitory activity than the related **5d** or **5g** compounds (Table 3). Compounds **4a-e**, bearing the 2-chlorobenzyl group at 2-position, even when variously combined with other O-substituents, did not show good inhibitory activity. In analogy to our previous studies, only the derivatives **2c, 2d, 2g, 2h**, which displayed a significantly improved activity with respect to the already reported compounds [18], were tested as $P2Y_{12}$ antagonists in a functional assay [20]. Surprisingly, there was no measurable antagonism for a known agonist 2-methylthioadenosine diphosphate (2-MeSADP) at 10^{-4}M as shown in Fig. (3). Since these compounds were able to inhibit platelet aggregation, we hypothesized a possible $P2Y_1$ antagonist activity, but the results in a binding assay using [^{3}H]2-MeSADP did not support this mechanism either [21] (Fig. 4). Thus, the platelet antiaggregatory activity of our favored compounds, structurally related to our previous series [18], seemed not due to a specific interaction with the P2 purinergic receptors, but to another kind of interaction with the platelets, that we will intend to investigate in future.

In order to increase the activity profile of the series **2a-j**, **3a-j**, **4a-e**, and **5a-j**, we hypothesized that a different relationship between the substituents on the pyrimidine nucleus, our supporting scaffold, could give better results. For this reason, we decided to change the pyrimidine nucleus 6-amino-2-thio-3H-pyrimin-4-one **(7**) with the ethyl-4-amino-2-thiopyrimidine-5-carboxylate (**9**). As substituents on the thio function, we introduced arylalkyl groups predominantly bearing hydrophilic moieties. We used 3-amino-benzoic acid as common substituent at the carboxyl function at 5-position of compound **9** with the purpose to have a free acid group and an aromatic ring in the structure that seemed important for the activity. Unfortunately, these new compounds (**6a-i**), tested at 10^{-4}M, did not produce a relevant increase of the inhibitory activities of platelet aggregation induced by

10–6 M ADP respect to derivatives **2a-j** or **3a-j**. Moreover, compounds **6a-i** did not display interesting differences in inhibiting platelet aggregation induced by different ADP concentrations. This evidence could support the hypothesis of a different interaction with the platelets to be investigated (Table 4).

As matter of fact, the structures **6a-i**, designed with the purpose of increasing the inhibitory activity through various interactions, displayed much lower activity, showing that our hypothesis about a different distribution of the substituents on the common pyrimidine core failed. None of these compounds were tested in $P2Y_{12}$ or $P2Y_1$ receptors binding as-says because of their low platelet antiaggregatory activity. From these data, we have to conclude that the different distribution of the substituents on the pyrimidine nucleus of compounds (**6a-i**) with respect to the previous structures (**2a-j**) was not positive in terms of activity, and simpler structures (**2a-j**) gave better results.

CONCLUSION

In conclusion, we can assume that a substituted pyrimidine nucleus as basic structure to develop agents that inhibit platelet aggregation could represent a good starting point. The 6-amino-2-thio-3*H*-pyrimidin-4-one (**7**) as common core displayed greater inhibitory efficacy than ethyl-4-amino-2-thiopyrimidine-5-carboxylate (**9**). Among the substituents of the base structure **7**, small alkyl chains bearing an amino group as thio substituents associated with arylalkyl moieties at 4-position, displayed the greater inhibitory activity on ADP-induced platelet aggregation. Some compounds of these new series displayed a slightly greater ability to inhibit ADP-induced platelet aggregation with respect to our former lead compound **1** [18], confirming that the pyrimidine nucleus **7**, opportunely substituted, can be considered a common feature for developing platelet aggregation inhibitors. Unfortunately, $P2Y_{12}$ and $P2Y_1$ binding as-says of the more interesting compounds did not give positive results, meaning that the platelet antiaggregatory activity was not due to an interaction with these two receptors but, probably, to some yet undetermined interactions. In fact, it must be emphasized that concentrations of all our compounds lower than 10^{-4}M displayed no or negligible inhibitory activity on platelet aggregation. Therefore, we cannot rule out the hypothesis that at least part of the observed inhibitory effects are due to an unknown mechanism of interaction.

Nevertheless, we think that the more interesting compounds in the series of **2a-j**, deserve further studies in the attempt to increase their inhibitory activity, in particular to better understand a possible mechanism of action, which could be independent of purinergic receptors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

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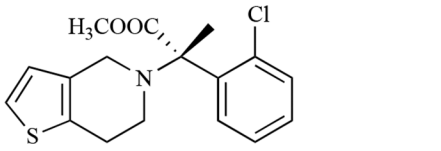
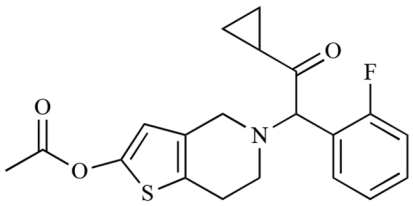
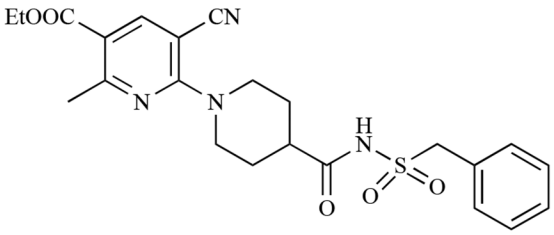


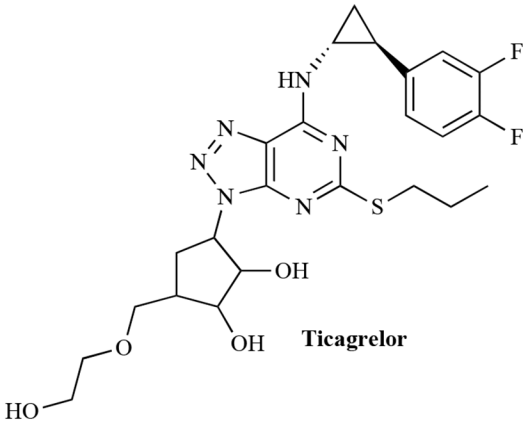
Clopidogrel



Prasugrel



AZD1283



Ticagrelor

Fig. (1).
Main $P2Y_{12}$ receptor antagonists.

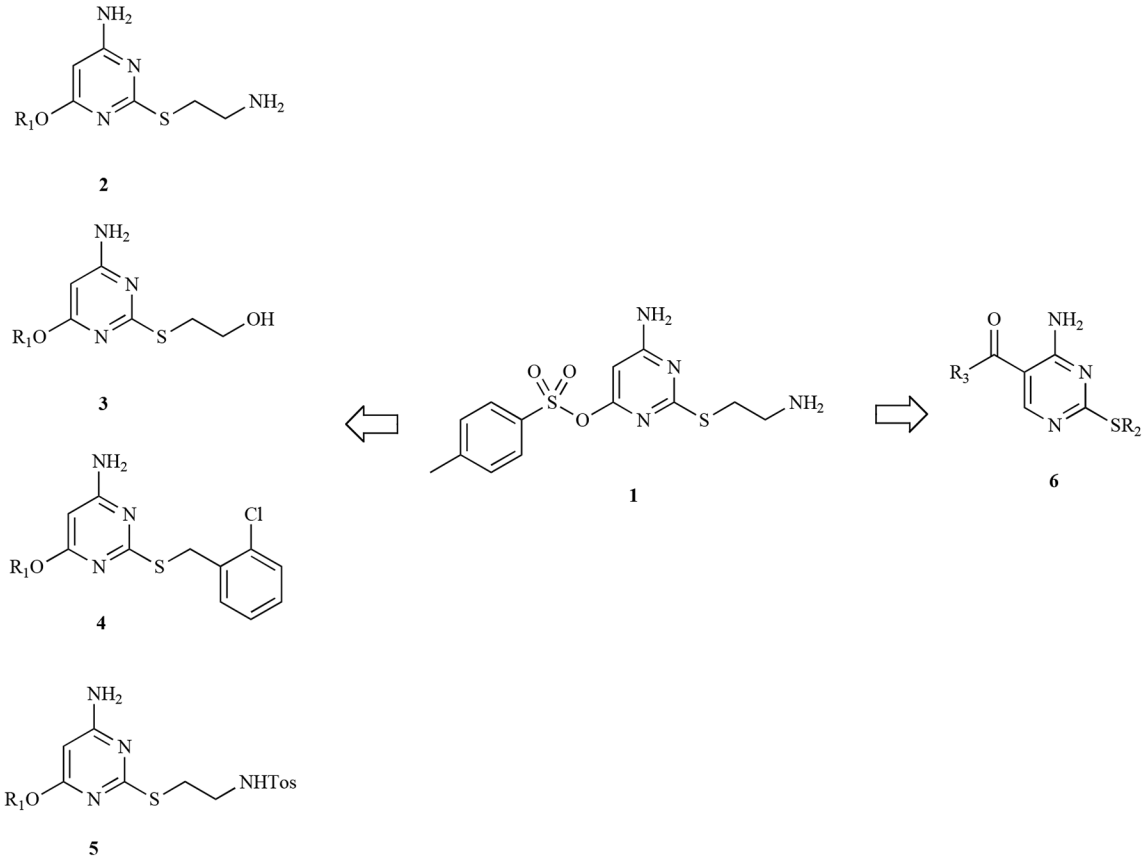


Fig. (2).
New projected and synthesized compounds.

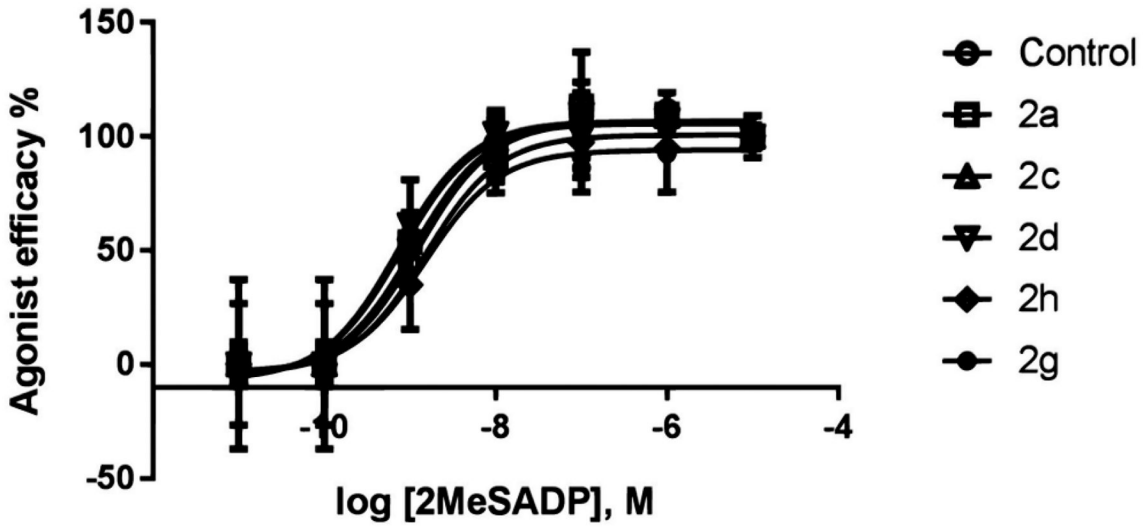


Fig. (3).

$P2Y_{12}$ functional assay. The compounds did not right-shift the agonist concentration-reponse curve. EC_{50} values of 2MeSADP, nM, were calculated to be 1.10 ± 0.21, 1.11 ± 0.16, 0.91 ± 0.22, 0.83 ± 0.23, 1.28 ± 0.31, 1.32 ± 0.28 for control, in the presence of 10^{-4}M **2a**, **2d**, **2c**, **2g**, **2h**, respectively.

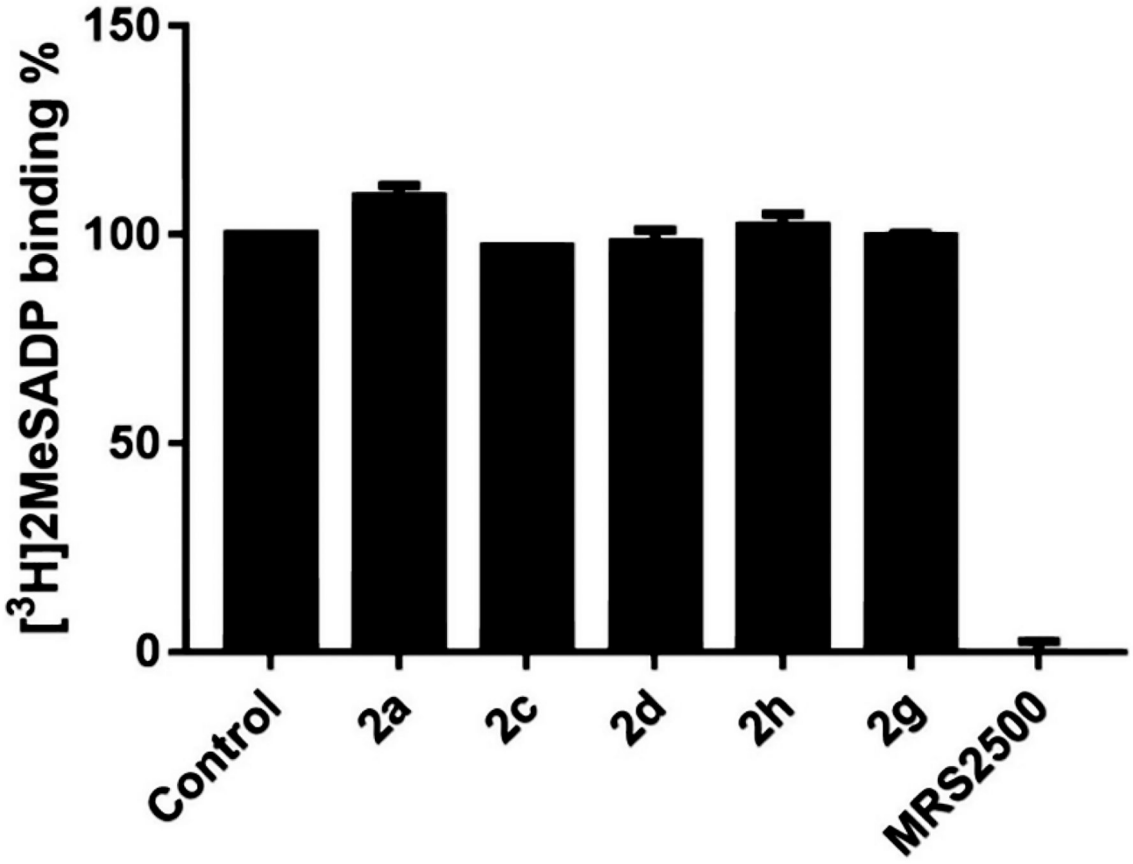
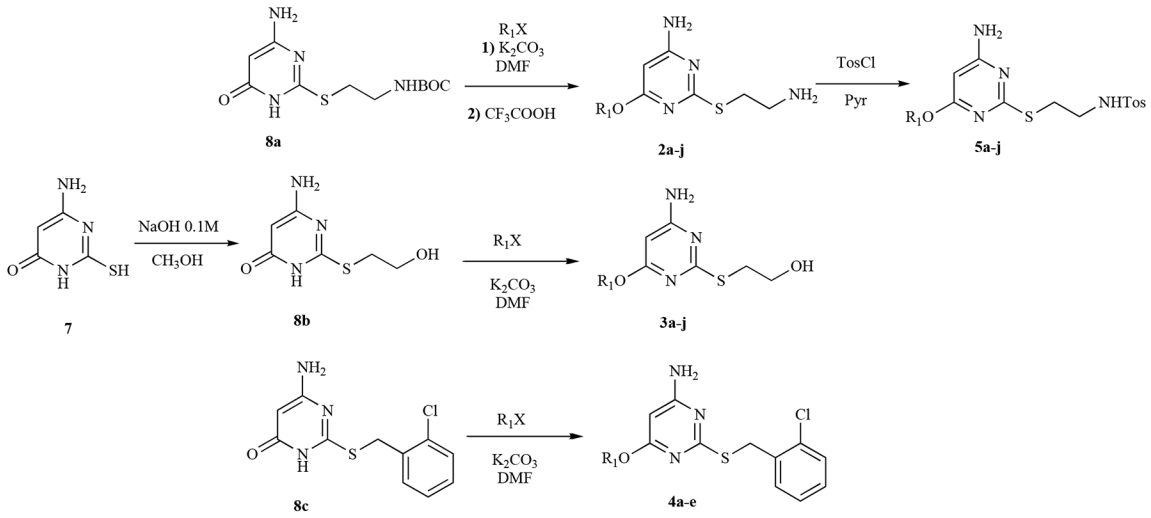


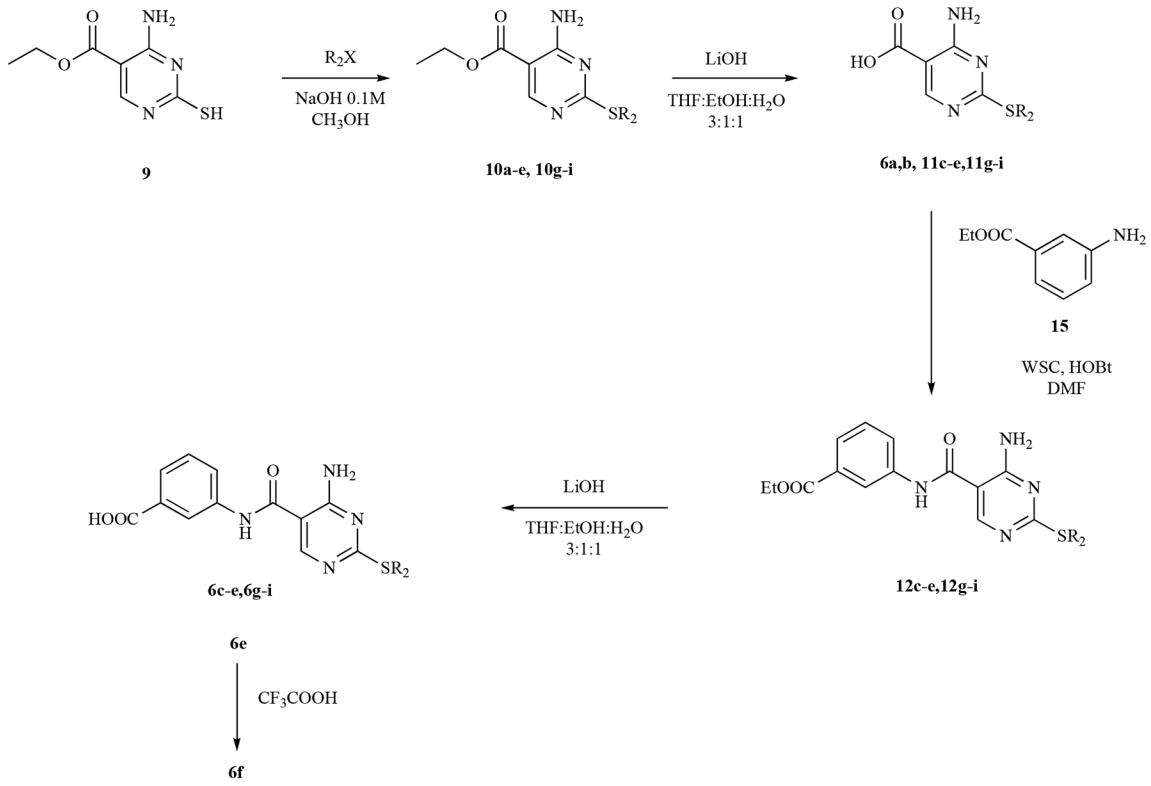
Fig. (4).

P2Y1 binding assay. Compounds were tested at 10^{-4}M, and except for MRS2500 (100% inhibition), all displayed <10% specific binding inhibition.



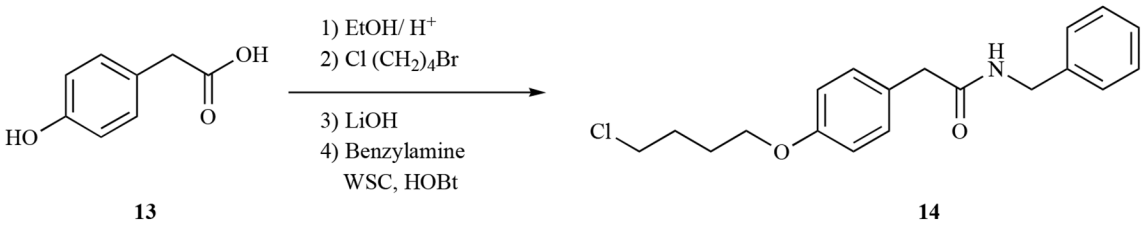
Scheme 1.

Synthesis of compounds **2a-j, 3a-j, 4a-e, 5a-j**.



Scheme 2.

Synthesis of compounds **6a-e, 6g-i**.



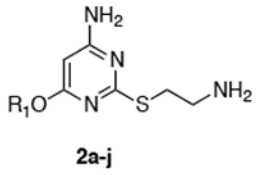
13 **14**

Scheme 3.

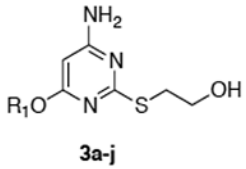
Synthesis of N-benzyl-2-(4-(4-chlorobutoxy)phenyl)acetamide **14**.

Table 1.

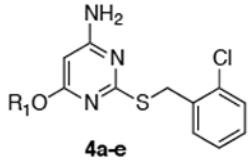
List of different substituents in compounds 2a-j, 3a-j, 4a-e, 5a-j.



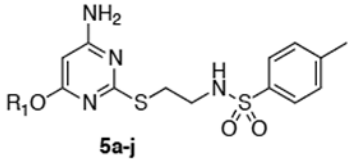
2a-j



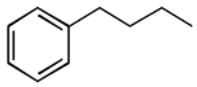
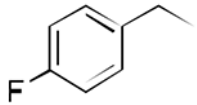
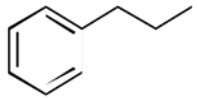
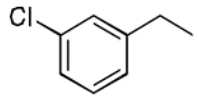
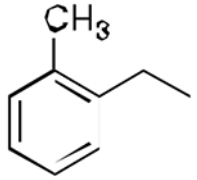
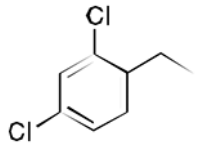
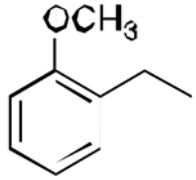
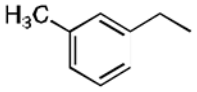
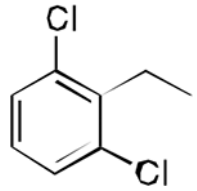
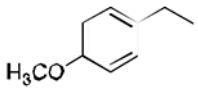
3a-j



4a-e



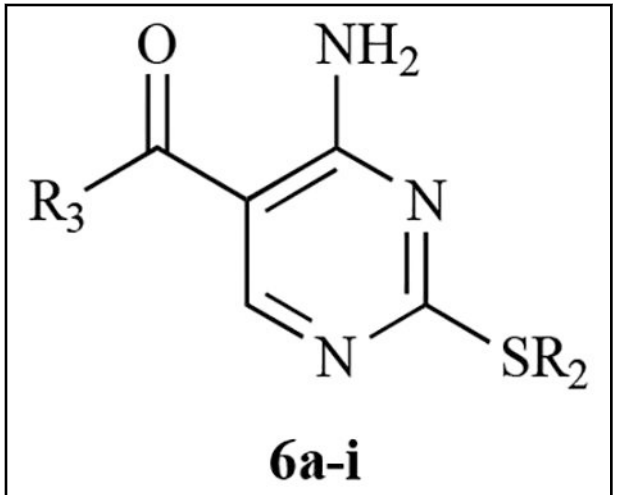
5a-j

Compound	R_1	Compound	R_1	Compound	R_1
2a, 3a, 5a		**2f, 3f, 5f**		**4a**	
2b, 3b, 5b		**2g, 3g, 5g**		**4b**	CH_3
2c, 3c, 5c		**2h, 3h, 5h**		**4c**	
2d, 3d, 5d		**2i, 3i, 5i**		**4d**	

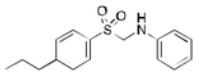
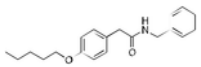
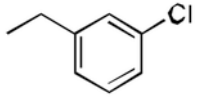
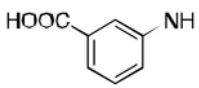
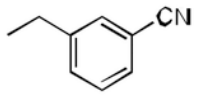
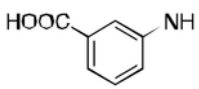
Compound	R_1
2e, 3e, 5e	
2j, 3j, 5j	
4e	
5a-j	CH_2COOH

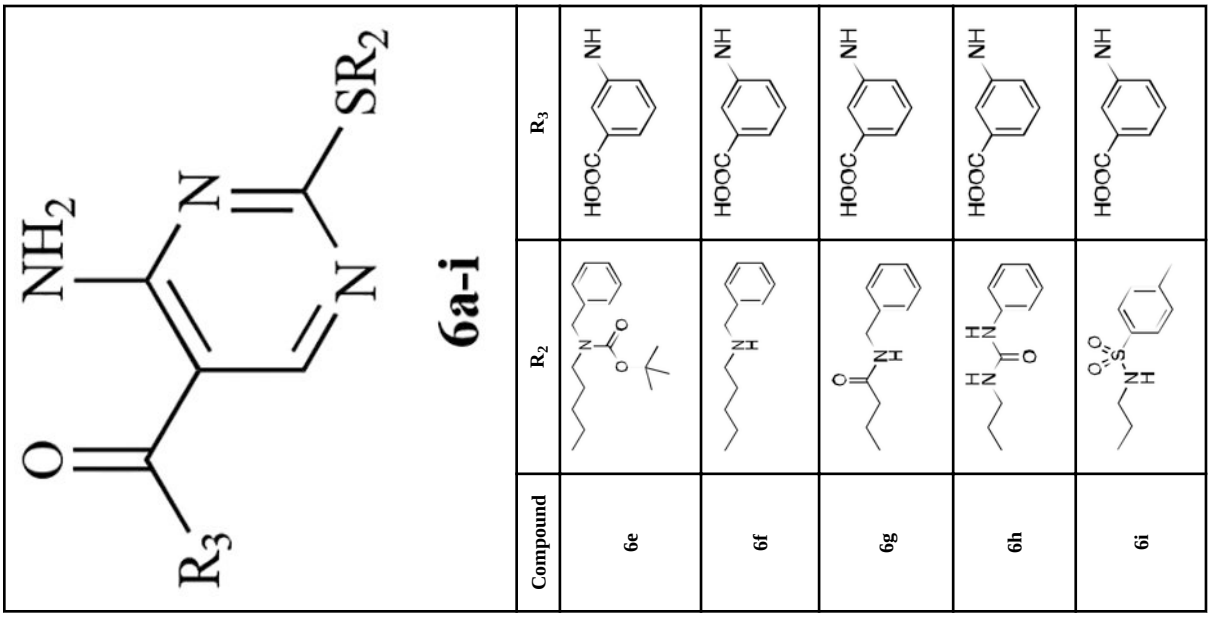
Table 2.

List of substituents of compounds 6a-i.



6a-i

Compound	R_2	R_3
6a		OH
6b		OH
6c		
6d		



Compound	R_2	R_3
6e		
6f		
6g		
6h		
6i		

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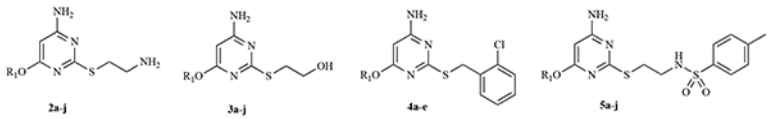
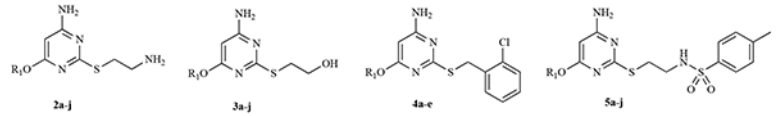
Table 3.

Effect of different compounds on platelet aggregation induced by two different concentrations of ADP.



Platelet Aggregation (% of control)*			
Compound	**R_1**	**ADP 10^{-5}**	**ADP10^{-6}**
2a	3-Ph$CH_2CH_2CH_2$	88	24
2b	2-PhCH_2CH_2	96	47
2c	2-CH_3-Benzyl	72	9
2d	3-CH_3-Benzyl	79	27
2e	4-Br-Benzyl	85	38
2f	4-F-Benzyl	86	36
2g	3-Cl-Benzyl	79	21
2h	2,4-diCl-Benzyl	65	13
2i	2,6-diCl-Benzyl	81	43
2j	3,5-diCH_3-Benzyl	77	31
3a	3-Ph$CH_2CH_2CH_2$	103	90
3b	2-PhCH_2CH_2	94	70
3c	2-CH_3-Benzyl	97	81
3d	3-CH_3-Benzyl	100	82
3e	4-Br-Benzyl	102	72
3f	4-F-Benzyl	95	67
3g	3-Cl-Benzyl	102	76
3h	2,4-diCl-Benzyl	100	58
3i	2,6-diCl-Benzyl	99	69
3j	3,5-diCH_3-Benzyl	94	73
4a	CH_2CH_2OH	101	85

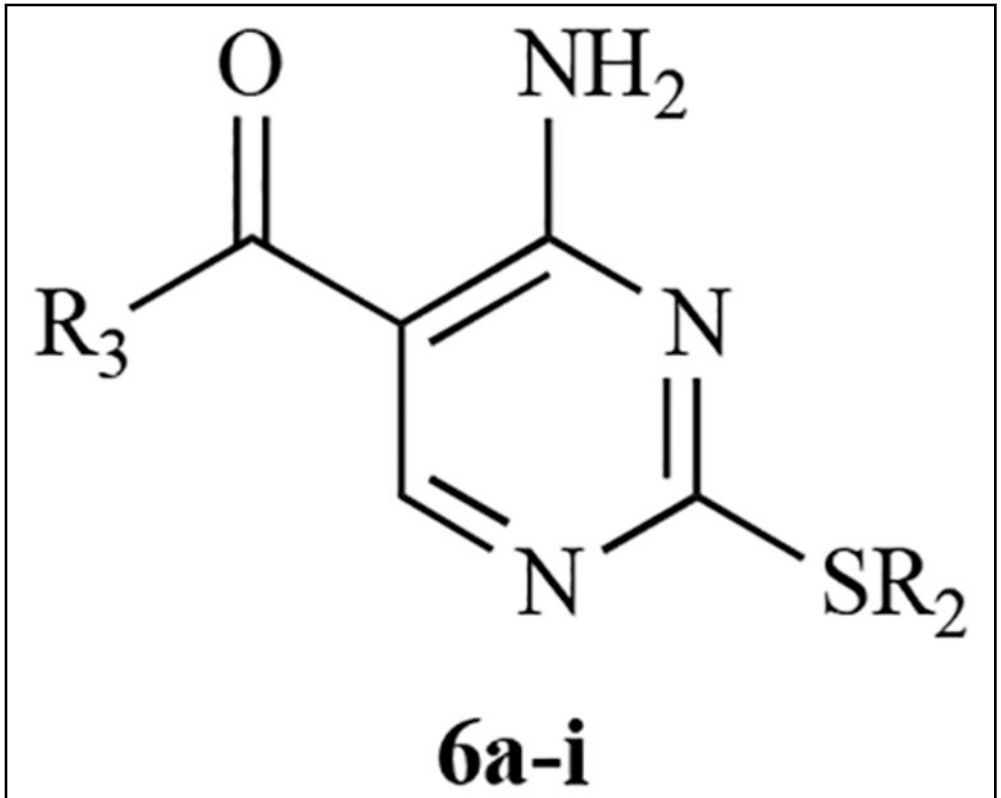


Platelet Aggregation (% of control)*			
Compound	**R_1**	**ADP 10^{-5}**	**ADP10^{-6}**
4b	CH_3	101	64
4c	2-OCH_3-Benzyl	102	98
4d	4-OCH_3-Benzyl	103	82
4e	CH_2COOH	99	85
5a	3-$PhCH_2CH_2CH_2$	75	65
5b	2-$PhCH_2CH_2$	90	67
5c	2-CH_3-Benzyl	87	51
5d	3-CH_3-Benzyl	79	86
5e	4-Br-Benzyl	88	90
5f	4-F-Benzyl	105	93
5g	3-Cl-Benzyl	82	66
5h	2,4-diCl-Benzyl	76	69
5i	2,6-diCl-Benzyl	91	60
5j	3,5-diCH_3-Benzyl	103	84

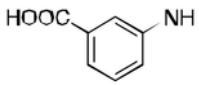
*Platelet aggregation observed in the presence of each compound was expressed as % of platelet aggregation that was measured in the presence of the vehicle (mean values of at least 2 experiments).

Table 4.

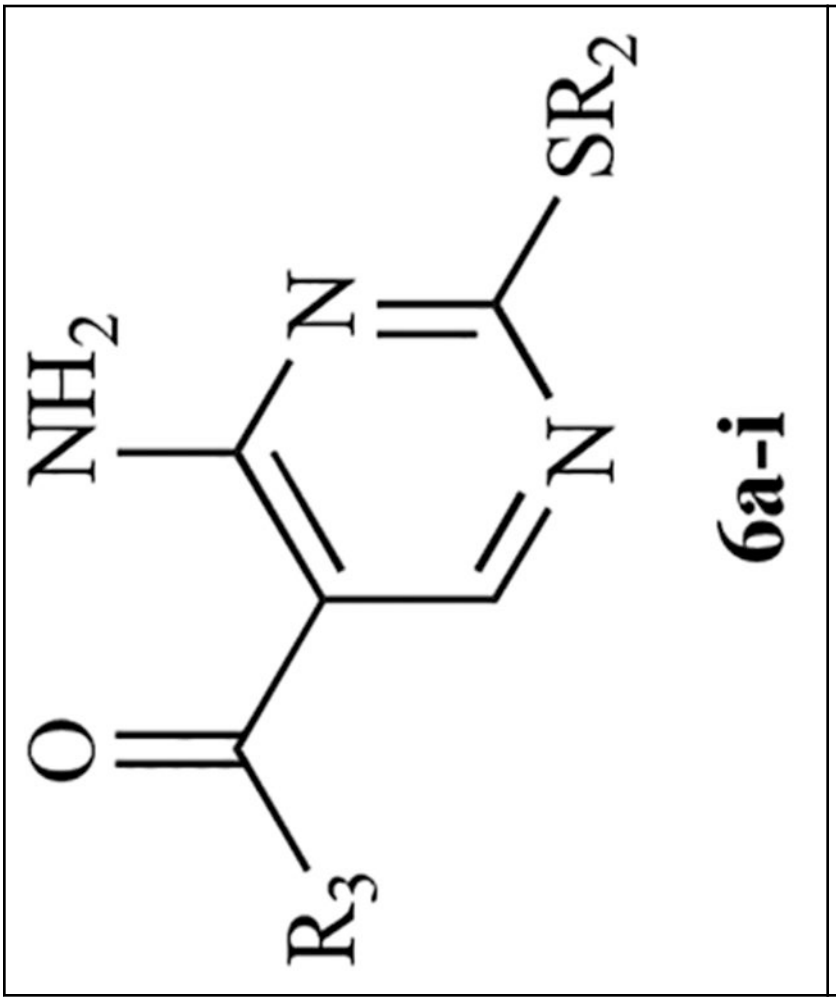
Effect of different compounds on platelet aggregation induced by two different concentrations of ADP.



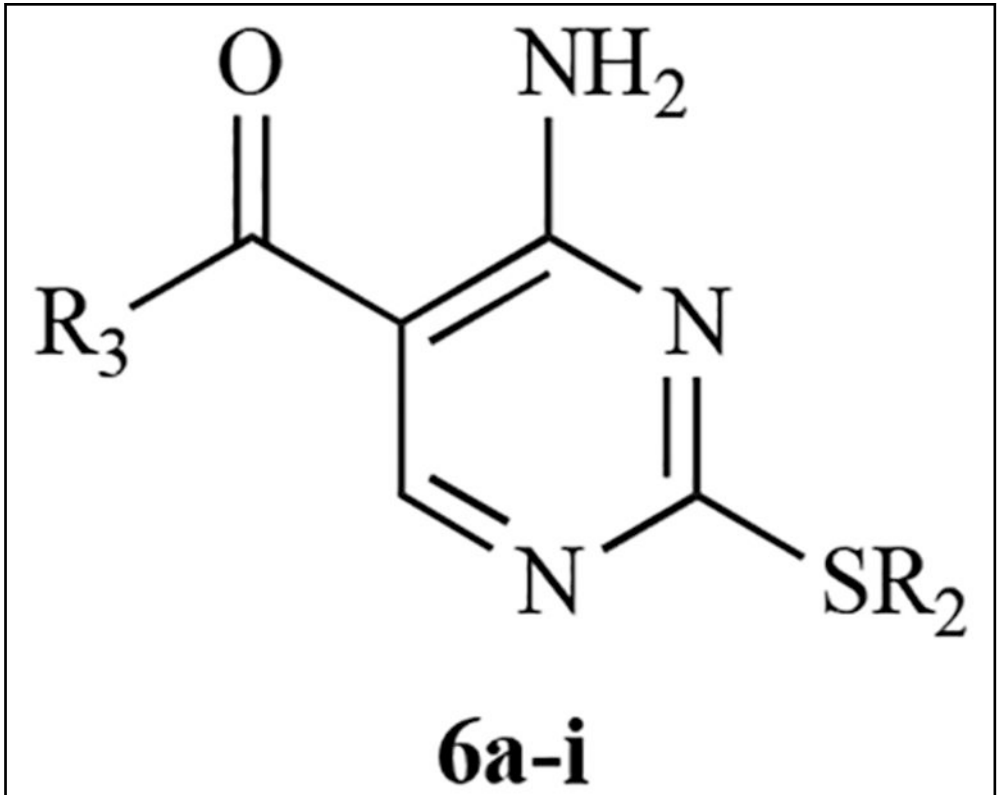
6a-i

Platelet Aggregation (% of control)*				
Compound	**R_2**	**R_3**	**ADP 10^{-5}**	**ADP10^{-6}**
6a	4(*N*-Benzylsulfamoyl)-phenethyl	OH	49	52
6b	4-(4-(2-(benzylamino)-2-oxoethyl)phenoxy)butyl	OH	51	47
6c	3-Cl-Benzyl		52	56

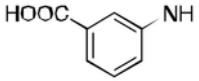
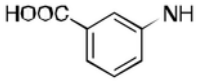
Compound	R_2	R_3	Platelet Aggregation (% of control)* ADP 10^{-5}	ADP10^{-6}
6d	3-CN-Benzyl		48	57
6e	4-(benzyl(*tert*-butoxycarbonyl)amino)butyl		54	58



Compound	R_2	R_3	Platelet Aggregation (% of control)* ADP 10^{-5}	ADP10^{-6}
6f	4-(benzylamino)butyl		53	50
6g	3-(benzylamino)-3-oxopropyl		56	55



6a-i

Platelet Aggregation (% of control)*				
Compound	**R_2**	**R_3**	**ADP 10^{-5}**	**ADP10^{-6}**
6h	2-(3-phenylureido)ethyl		44	59
6i	(2-(4-methylphenylsulfonamido)ethyl		48	57

*Platelet aggregation observed in the presence of each compound was expressed as % of platelet aggregation that was measured in the presence of the vehicle (mean values of at least 2 experiments).