Notes

Platelet Aggregation Inhibitors. 7.[1] S-Substituted 2-Thioadenosine 5′-Monophosphates

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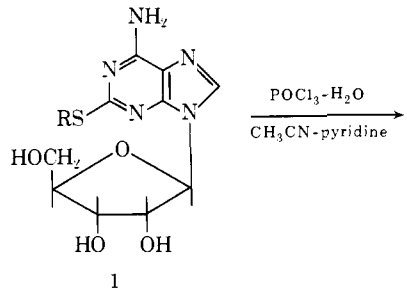
Aggregation of blood platelets or formation of platelet thrombi is of primary importance in arterial thrombogenesis.[2] Adenosine 5′-diphosphate (ADP) is known to induce platelet aggregation both *in vitro*[3] and *in vivo*.[4] Adenosine is a powerful inhibitor of ADP-induced platelet aggregation,[5] and certain derivatives of adenosine such as 2-chloroadenosine,[6] N^6-substituted adenosines,[7,8] and S-substituted 2-thioadenosines[1] are also potent inhibitors. Adenosine 5′-monophosphate (AMP) has been known to be a less effective inhibitor,[3] and 2-chloroadenosine 5′-monophosphate[9] and certain N^6-substituted adenosine 5′-monophosphates[8] are almost inactive. 2-Methylthioadenosine 5′-monophosphate, however, is much more potent than the original nucleoside, 2-methylthioadenosine, and has been evaluated by Michael, *et al.*,[10] as an antithrombotic agent without undesirable side effects. In the present study, some additional S-substituted 2-thioadenosine 5′-monophosphates were prepared and tested as inhibitors of platelet aggregation mediated by ADP and collagen.



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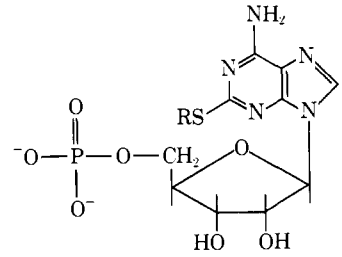

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a, R = $CH_3(CH_2)_4$–
b, R = $C_6H_5CH_2$–
c, R = CH_2=$CHCH_2$–

2-*n*-Amylthioadenosine (**1a**), 2-benzylthioadenosine (**1b**), and 2-allylthioadenosine (**1c**) which were representative of the S-substituted 2-thioadenosines reported in a previous paper[1] were directly phosphorylated at their 5′-hydroxyl functions by reaction with aqueous $POCl_3$ in the presence of pyridine and acetonitrile.[11] The structures of the products isolated as barium salts (yield, 10-20%) were identified as 2-*n*-amylthioadenosine 5′-monophosphate (2-*n*-amylthio-AMP, **2a**), 2-benzylthioadenosine 5′-monophosphate (2-benzylthio-AMP, **2b**), and 2-allylthioadenosine 5′-monophosphate (2-allylthio-AMP, **2c**) by ultraviolet absorption spectrum, paper chromatography, paper electrophoresis (Table I), elemental analysis, and dephosphorylation by *Crotalus atrox* 5′-nucleotidase.

2-*n*-Amylthio-AMP (**2a**), 2-benzylthio-AMP (**2b**), and 2-allylthio-AMP (**2c**) were tested as inhibitors of ADP-induced platelet aggregation on samples of buffered platelet-rich plasma prepared from rabbit citrated blood and platelet-rich plasma from human citrated blood. Inhibition percentages of platelet aggregation by **2a-c** compared to those by adenosine and AMP are listed in Table II. On rabbit platelet aggregation ammonium salts of **2a** and **2c** dissolved in saline showed potent inhibitory effect comparable to adenosine at any concentration; these exhibited 20-80% inhibition at 10^{-6}-10^{-4} *M*, whereas the ammonium salt of **2b** showed 50% inhibition at 10^{-4} *M* and was inactive at below 10^{-5} *M*. Thus, the phosphorylated nucleosides **2a** and **2c** were more effective inhibitors of ADP-induced aggregation than the original nucleosides **1a** and **1c**.[1]

Considerable species variation exists in the response of platelet aggregation to inhibition by 2-methylthio-AMP;[10] it was 2.4 times more potent than adenosine against sheep platelet aggregation while it was 8.6 times less potent than adenosine against human platelet aggregation. When **2a** and **2c** were tested as inhibitors of human platelet aggregation, the inhibitory effect of these compounds was also comparable to that of adenosine. Hence, **2a** and **2c** might be considered more powerful inhibitors of ADP-induced aggregation than 2-methylthio-AMP with respect to human species.

Compounds **2a** and **2c** were preincubated with buffered rabbit platelet-rich plasma at 37° for the longer intervals

Table I. Uv Spectra, Paper Chromatography, and Paper Electrophoresis of S-Substituted 2-Thioadenosine 5′-Monophosphates

Compound	Uv, λ max, nm (ε × 10^{-3})			Paper chromatography		Paper electrophoresis[c]
	pH 1	H_2O	pH 13	$R_{f(1)}$[a]	$R_{f(2)}$[b]	
2-*n*-Amylthio-AMP (**2a**) barium salt	272 (16.6)	278.5 (15.5)	278.5 (15.5)	0.75	0.67	+0.49
2-Benzylthio-AMP (**2b**) barium salt	273 (16.6)	277 (16.2)	278 (15.4)	0.66	0.56	+0.48
2-Allylthio-AMP (**2c**) barium salt	272 (16.2)	278 (15.4)	278 (16.4)	0.56	0.38	+0.48
2-*n*-Amylthioadenosine (**1a**)[d]	272 (16.1)	278 (14.6)	279 (14.7)	0.93	0.93	
2-Benzylthioadenosine (**1b**)[d]	272.5 (16.4)	278 (15.9)	278 (15.9)	0.91	0.91	
2-Allylthioadenosine (**1c**)[d]	272 (16.1)	278 (15.0)	278 (14.9)	0.86	0.87	
AMP				0.29	0.20	+1.00
Adenosine 2′,3′-monophosphate				0.34	0.28	
ADP				0.21	0.13	+1.27

[a]Solvent: *n*-PrOH–concentrated NH_4OH–H_2O (20:10:3). [b]Solvent: *i*-PrOH–concentrated NH_4OH–H_2O (7:1:2). [c]Mobility relative to that of AMP. [d]Reference 1.

Table II. Inhibition of ADP-Induced Platelet Aggregation by S-Substituted 2-Thioadenosine 5′-Monophosphates

Compound	Molar concn	% inhibition[a] Rabbit plasma (10^{-5} *M* ADP)	% inhibition[a] Human plasma (3×10^{-6} *M* ADP)
2-*n*-Amylthio-AMP (**2a**) ammonium salt	8×10^{-5}	80	
	8×10^{-6}	54	84
	8×10^{-7}	22	54
2-Benzylthio-AMP (**2b**) ammonium salt	1.2×10^{-4}	50	
	1.2×10^{-5}	7	31
	1.2×10^{-6}	0	
2-Allylthio-AMP (**2c**) ammonium salt	9×10^{-5}	80	
	9×10^{-6}	59	86
	9×10^{-7}	37	31
AMP disodium salt	10^{-4}	52	
	10^{-5}	35	
	10^{-6}	5	
Adenosine	10^{-4}	71	
	10^{-5}	61	94
	10^{-6}	37	41

[a] Buffered platelet-rich plasma from citrated rabbit blood or platelet-rich plasma from citrated human blood (1.0 ml) was incubated at 37° for 3 min with 10 μl of a solution of each of the test compounds in saline and challenged with ADP in saline. Per cent inhibition by a test compound was calculated by dividing the maximum deflection of the optical density curve by that observed with control solvent (saline). As the sensitivity of platelets to ADP varied from preparation to preparation, more than two experiments were carried out with different preparation of platelet-rich plasma in the cases of rabbit and human and inhibition percentages listed in the table were representative ones under the same conditions.

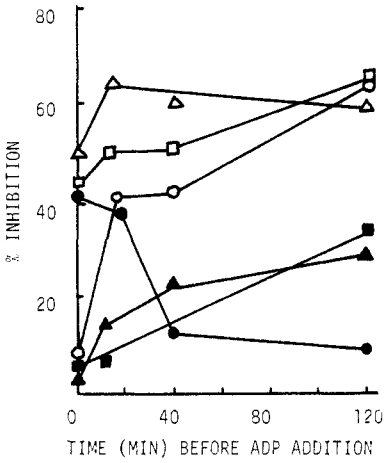

Figure 1. Effect of preincubation of S-substituted 2-thioadenosine 5′-monophosphates with rabbit platelet-rich plasma on ADP-induced platelet aggregation. Buffered platelet-rich plasma (1.0 ml) was incubated at 37° with the test sample in saline and after the indicated period it was challenged with 10^{-5} *M* ADP. 2-*n*-Amylthio-AMP (2a) ammonium salt, 8×10^{-6} *M* (□); 2-allylthio-AMP (2c) ammonium salt, 9×10^{-6} *M* (△); AMP, 10^{-4} *M* (○); 2-*n*-amylthioadenosine, 10^{-5} *M* (■); 2-allylthioadenosine, 10^{-5} *M* (▲); and adenosine, 10^{-5} *M* (●).

(up to 120 min) before the addition of ADP (Figure 1). At 10^{-5} *M* inhibition percentages of **2a** and 2c were maintained around 50%, whereas those of the dephosphorylated nucleosides 1a and 1c gradually increased for 120 min but not exceeded 30% and that of adenosine gradually decreased below 10%. The results indicated that **2a** and 2c were powerful inhibitors characterized by long-lasting activity. Inhibition percentages of AMP at 10^{-4} *M* increased as the preincubation proceeded, and the results agreed with that of Born and Cross[3] who used human platelet-rich plasma. There have been some discussions about the inhibitory effect of AMP; some reported that it was an inhibitor by itself[12,13] while others reported that the inhibitory effect of AMP was due to the hydrolyzed product, adenosine.[14,15] In our assay systems it was not known whether AMP was an inhibitor by itself or whether the hydrolyzed product, adenosine, was an actual inhibitor. Compounds 2a and 2c could be dephosphorylated by plasma phosphatase or 5′-nucleotidase, but the dephosphorylated nucleosides 1a and 1c were less potent at 10^{-5} *M* than 2a and 2c. This suggested that 2a and 2c may act directly on platelet without dephosphorylation to 1a and 1c or may be more rapidly taken up into platelet membrane than 1a and 1c thereby giving greater inhibition upon subsequent dephosphorylation.

S-Substituted 2-thio-AMP's **2a**-c were also tested as inhibitors of collagen-induced rabbit platelet aggregation and were found inactive. Thus, ammonium salts of **2a**-c preincubated for 3 min with buffered platelet-rich plasma inhibited less than 10% of collagen-induced aggregation at 10^{-4} *M*, whereas adenosine inhibited 93% at 10^{-4} *M* and 42% at 10^{-5} *M*, and AMP inhibited 20% at 10^{-4} *M*. Nucleosides 1a and 1c were effective at 10^{-4} *M* against both ADP- and collagen-induced aggregation,[1] whereas the nucleotides 2a and 2c were ineffective at the same concentration against collagen-induced aggregation. This seemed interesting to us since most agents that inhibit ADP-induced aggregation inhibit collagen-induced aggregation.[2]

In conclusion, 2-*n*-amylthio-AMP (**2a**) and 2-allylthio-AMP (2c) were found to be potent inhibitors of ADP-induced rabbit and human platelet aggregation. The degree of inhibition by 2a and 2c was comparable to that of adenosine in both species and the inhibitory effect was characterized by long-lasting activity.

Experimental Section

Methods. Where analyses were indicated only by symbols of the elements, analytical results obtained for those elements were within ±0.4% of the theoretical values. Uv spectra were measured with a Hitachi recording spectrophotometer EPS-3T. Paper chromatography was performed using Tōyō Roshi No. 51A paper in an ascending technique with solvent systems (1) *n*-PrOH–concentrated NH_4OH–H_2O (20:10:3) and (2) *i*-PrOH–concentrated NH_4OH–H_2O (7:1:2). Paper eletrophoresis was carried out in 0.05 *M* phosphate buffer (pH 7.5) at 1000 V/10 cm and for 1 hr, and the mobility was represented by a relative value to that of AMP. Determination of whole phosphorus was done according to the method of Allen[16] and that of inorganic phosphate was according to the method of Fiske and Subbarow.[17] Sodium metaperiodate and benzidine spray test was used to confirm the presence of the 2′,3′-*cis*-glycol function.[18]

Dephosphorylation by 5′-nucleotidase was performed as follows. A mixture of about 500 μg of the test sample dissolved in 1 ml of H_2O containing 10% DMSO, 2.4 ml of 0.05 *M* Tris-HCl (pH 7.5), 0.2 ml of 0.018 *M* $MgCl_2$, and 0.2 ml of a solution of *Crotalus atrox* venom (Sigma Chemical Co., Ltd.) (0.5 mg/ml of Tris-HCl

buffer) was incubated at 37° for 15 hr and was added to 0.2 ml of 55% cold trichloroacetic acid. After centrifugation at 3000 rpm for 10 min, the supernatant (1 ml) was submitted to the inorganic phosphate determination. Under the conditions, AMP and ADP liberated 0.95 and 1.96 mol of inorganic phosphate, respectively, while adenosine 2',3'-monophosphate liberated less than 0.04 mol of inorganic phosphate.

2-*n*-Amylthioadenosine 5'-Monophosphate (2a). 2-*n*-Amylthioadenosine (**1a**),[1] 92.5 mg (0.25 mmol), was suspended in 0.25 ml of acetonitrile and 0.10 ml (1.2 mmol) of pyridine, and the mixture was cooled to 0°. To the suspension was added 0.10 ml (1.1 mmol) of $POCl_3$ and 0.010 ml (0.5 mmol) of H_2O, and the homogeneous mixture was stirred at 0° for 2 hr. It was poured into 5 ml of ice-water and was adjusted to pH 1 with 2 *N* HCl to afford a white precipitate. The precipitate was separated by centrifugation at 2000 rpm for 10 min and was redissolved in 0.5 ml of concentrated NH_4OH and submitted to preparative paper chromatography using solvent 1. Three bands (R_f 0.51, 0.42, and 0.29) were detected on the chromatogram by uv ray. The fastest moving major band (R_f 0.51) was cut out and extracted with 0.05 *N* NH_4OH and the extract was evaporated to a small volume. In order to eliminate minor contaminants and impurities it was purified again by the same preparative chromatography. The single band was extracted by 0.05 *N* NH_4OH and was evaporated to dryness and redissolved in about 2.0 ml of H_2O, which was subsequently filtered through a millipore filter (0.22 μ). To the filtrate was added 35 mg (0.08 mmol) of BaI_2 dissolved in 4 ml of EtOH. The white precipitate was separated by centrifugation at 3000 rpm for 10 min and washed with H_2O-EtOH (1:2) twice and finally with EtOH. It was dried *in vacuo* at 50° for 24 hr over P_2O_5. 2-*n*-Amylthio-AMP (2a) barium salt was obtained in a yield of 13.5% (21.0 mg) based on **1a**. *Anal.* ($C_{15}H_{22}O_7N_5SPBa\cdot 2H_2O$) C, H, N, P. The content of inorganic phosphate was less than 0.01 mol. Labile phosphate by 5'-nucleotidase was 0.995 mol (theoretical, 1.00 mol). The sodium metaperiodate-benzidine test confirmed the presence of the 2',3'-*cis*-glycol function.

2-Benzylthioadenosine 5'-Monophosphate (2b). 2-Benzylthioadenosine (**1b**),[1] 105.5 mg (0.25 mmol), was suspended in 0.25 ml of acetonitrile and 0.10 ml of pyridine. The mixture was cooled to 0° and to this was added 0.10 ml of $POCl_3$ and 0.010 ml of H_2O. After the mixture was stirred at 0° for 2 hr, it was mixed with 5 ml of ice-water and subsequently with 0.5 ml of 2 *N* HCl. The white precipitate which separated was dissolved in concentrated NH_4OH and submitted to preparative paper chromatography as described above. Three bands (R_f 0.49, 0.40, and 0.26) were detected on the chromatogram. The major band (R_f 0.49) was extracted with 0.05 *N* NH_4OH and purified again by the chromatography. The barium salt of 2-benzylthio-AMP (**2b**) was obtained in a yield of 12.5% (20.0 mg) based on **1b**. *Anal.* ($C_{17}H_{18}O_7N_5SPBa\cdot 2H_2O$) C, H, N, P. The content of inorganic phosphate was less than 0.01 mol. Labile phosphate by 5'-nucleotidase was 1.048 mol (theoretical, 1.00 mol). The sodium metaperiodate-benzidine test confirmed the presence of the 2',3'-*cis*-glycol function.

2-Allylthioadenosine 5'-Monophosphate (2c). 2-Allylthioadenosine (**1c**),[1] 374 mg (1.0 mmol), was suspended in 1.0 ml of acetonitrile and 0.39 ml (4.8 mmol.) of pyridine. The mixture was cooled to 0° and to this was added 0.40 ml (4.4 mmol) of $POCl_3$ and 0.040 ml (2.0 mmol) of H_2O. After the mixture was stirred at 0° for 4 hr, it was subsequently added to 5 ml of cold water. A small amount of precipitate was removed by centrifugation, and the supernatant was acidified at pH 1 with 2 *N* HCl. It was then absorbed on a column of 20 g of active carbon. The column, washed well with H_2O, was eluted with 300 ml of 10% NH_4OH-EtOH (1:1). The effluent was evaporated *in vacuo* to dryness and submitted to preparative paper chromatography as described above. Three bands (R_f 0.46, 0.35, and 0.18) were detected on the chromatogram, and the relative amounts of the products corresponding to these three bands estimated by optical density (273 nm, pH 1) were 75, 8.5, and 16.5%, respectively. The major band (R_f 0.46) was cut out, extracted with 0.05 *N* NH_4OH, evaporated to dryness, and redissolved in 6.0 ml of H_2O. It was subsequently filtered through a millipore filter and the filtrate was added to 105 mg (0.25 mmol) of barium iodide dissolved in 12 ml of EtOH. The white precipitate which separated was collected by centrifugation. It was redissolved in 6.0 ml of H_2O, filtered, and precipitated by the addition of 12 ml of EtOH. Finally it was washed with EtOH twice and dried to afford 90.0 mg (yield, 15.5%) of 2-allylthio-AMP (2c) barium salt. *Anal.* ($C_{13}H_{16}O_7N_5SPBa\cdot 1.5H_2O$) C, H, N, P. The content of inorganic phosphate was less than 0.01 mol. Labile phosphate by 5'-nucleotidase was 0.997 mol (theoretical, 1.00 mol). The sodium metaperiodate-benzidine test confirmed the presence of the 2',3'-*cis*-glycol function. The products corresponding to the bands, R_f 0.35 and 0.18, must be the 5'-diphosphate analog and 2',3'-phosphorylated analog(s) examined by paper chromatography, paper electrophoresis, and the sodium metaperiodate-benzidine test.

Platelet Aggregation Test. Platelet aggregation studies were performed on buffered platelet-rich plasma from rabbit citrated blood and platelet-rich plasma from human citrated blood, using the optical density method reported before.[1] Platelet-rich plasma from a male rabbit was immediately buffered with an equal volume of isotonic barbital buffer (pH 7.3), and the mixture was used as buffered platelet-rich plasma.

As the barium salts of **2a-c** were insoluble in saline, ammonium salts were used in platelet aggregation studies. The ammonium salt of each nucleotide to be tested was prepared from the filtrate through the millipore filter of the NH_4OH extract of the spot corresponded to the nucleotide. Concentration of each nucleotide in saline was determined by optical density. The extract of filter paper by dilute NH_4OH did not contain any substances that affect platelet aggregation, and the inhibitory effect of the NH_4OH extract of 2a-c on paper chromatograms could be attributed to the ammonium salts of **2a-c**.

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References

(1) K. Kikugawa, H. Suehiro, and M. Ichino, *J. Med. Chem.*, **16**, 1381 (1973) (paper 6).
(2) A. Gaarder, J. Jonsen, S. Laland, A. Hellem, and P. A. Owren, *Nature (London)*, **192**, 531 (1961).
(3) G. V. R. Born and M. J. Cross, *J. Physiol.*, **168**, 178 (1963).
(4) G. V. R. Born, A. J. Honour, and J. R. A. Michell, *Nature (London)*, **202**, 761 (1964).
(5) G. V. R. Born and M. J. Cross, *Proc. Phys. Soc., London*, 29 (1962).
(6) G. V. R. Born, *Nature (London)*, **202**, 95 (1964).
(7) K. Kikugawa, K. Iizuka, Y. Higuchi, H. Hirayama, and M. Ichino, *J. Med. Chem.*, **15**, 387 (1972).
(8) K. Kikugawa, K. Iizuka, and M. Ichino, *ibid.*, **16**, 358 (1973).
(9) G. Gough, M. H. Maguire, and F. Michael, *ibid.*, **12**, 494 (1969).
(10) (a) F. Michael, M. H. Maguire, and G. Gough, *Nature (London)*, **222**, 1073 (1969); (b) British Patent 1,226,699 (1972); (c) U. S. Patent 3,678,162 (1972).
(11) S. Ouchi, T. Yamada, M. Kurihara, S. Seno, and T. Sowa, Japanese Patent 11464 (1968); *Chem. Abstr.*, **70**, 4549d (1969).
(12) M. A. Packham, N. G. Ardlie, and J. F. Mustard, *Amer. J. Physiol.*, **217**, 1009 (1969).
(13) M. A. Packham, M. A. Guccione, D. W. Perry, R. L. Kinlough-Rathbone, and J. F. Mustard, *ibid.*, **223**, 419 (1972).
(14) E. W. Salzman, D. A. Chambers, and L. L. Neri, *Nature (London)*, **210**, 167 (1966).
(15) M. C. Rozenberg and H. Holmsen, *Biochim. Biophys. Acta*, **155**, 342 (1968).
(16) R. J. L. Allen, *Biochem. J.*, **34**, 858 (1940).
(17) C. H. Fiske and Y. Subbarow, *J. Biol. Chem.*, **66**, 375 (1925).
(18) J. A. Cifonelli and F. Smith, *Anal. Chem.*, **26**, 1132 (1954).

Synthesis of 1,4-Bis(6-methoxy-8-quinolylaminoalkyl)piperazines as Potential Prophylactic Antimalarial Agents

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Malaria continues to be a problem, despite the years of research and screening of thousands of compounds. Today, drugs are available that interrupt the infection at its various stages, but clinically useful prophylactic anti-