Table I. Phosphoramidates Evaluated for *in Vitro* Antiplaque Activity

$$R_1R_2N{-}P(=O)(OR_3)_2$$

Compd	R_1	R_2	R_3	Reaction time,[a] hr: Reflux	Reaction time,[a] hr: Room temp	Yield, %[c]	Mp (solvent)[b] or bp (mm), °C	Formula	Analyses
5	H	C_6H_5	C_2H_5	0	16	85	94–96[d] (A/B)	$C_{10}H_{16}NO_3P$	C, H, N
6	H	4-ClC_6H_4	C_2H_5	1	0.5	50	76–78[e] (C)	$C_{10}H_{15}ClNO_3P$	C, H, N
7	H	3-$CF_3C_6H_4$	C_2H_5	0	144	42	137 (0.5)	$C_{11}H_{18}F_3NO_3P$	C, H, N
8	H	1-Naphthyl	C_2H_5	6	66	51	110–113 (D/E)	$C_{14}H_{15}NO_3P$	C, H, N
9[f]	H	1-Adamantyl	C_2H_5	1	1	58	97.5–102 (C)	$C_{14}H_{26}NO_3P$	C, H, N
10		$-(CH_2)_5-$	C_2H_5	2	0	53	110 (4)	$C_9H_{20}NO_3P \cdot 0.5H_2O$	C, H, N
11	H	n-$C_{14}H_{29}$	C_2H_5	2	0	24	47.5–50 (C)	$C_{18}H_{40}NO_3P$	C, H, N, P
12	H	C_6H_5	C_6H_5	2	17	54	128–130[g] (D/E)	$C_{18}H_{16}NO_3P$	C, H, N
13	H	4-ClC_6H_4	C_6H_5	1.5	16	32	115–118.5[h] (D/F)	$C_{18}H_{15}ClNO_3P$	C, H, N, P
14	H	3-$CF_3C_6H_4$	C_6H_5	1	16	15	89–93 (D/G)	$C_{19}H_{15}F_3NO_3P$	C, H, N
15[f]	H	1-Adamantyl	C_6H_5	1	16	46	125–127 (D/G)	$C_{22}H_{26}NO_3P$	C, H, N, P
16	H	n-$C_{12}H_{25}$	C_6H_5	2.5	0	46	62–63.5 (C/D)	$C_{24}H_{36}NO_3P$	C, H, N
17	H	n-$C_{14}H_{29}$	C_6H_5	2.5	0	55	65.5–67.5 (C/D)	$C_{26}H_{40}NO_3P$	C, H, N, P
18	H	Cyclohexyl	$CH_2C_6H_5$	0	22	42	76.5–80[i] (C/D)	$C_{20}H_{25}NO_3P$	C, H, N
19		$(C_2H_5O)_2P(O)N(CH_2CH_2)_2$	C_2H_5	2	0	58	Oil[j]	$C_{12}H_{28}N_2O_6P_2 \cdot 0.75H_2O$	C, H, N

[a] Length of reaction at reflux and/or room temperature. [b] Recrystallization solvents: A = ethanol; B = water; C = n-pentane; D = hexane; E = benzene; F = cyclohexane; G = 1,2-dichloroethane. [c] No attempt was made to optimize the yields. [d] Lit. mp 95.5–96.5°: H. McCombie, B. C. Saunders, and G. J. Stacey, *J. Chem. Soc.*, 380 (1945). [e] Lit. mp 76°: P. Otto, Ber., **28**, 616 (1895). [f] L. A. Cates, University of Houston, informed us subsequent to the completion of our synthetic work that he had independently prepared the adamantyl compounds for other purposes. [g] Lit. mp 128–129°: A. B. Foster, W. G. Overhead, and M. Stacey, *J. Chem. Soc.*, 980 (1951). [h] Lit. mp 116°: J. I. G. Cadogan and W. R. Foster, *ibid.*, 1079 (1957). [i] Lit.[8] mp 79–80°. [j] Purified by chromatography on silica gel (Woelm dry column grade) by eluting with C_6H_6–$CHCl_3$ (50:50) and 1% EtOH–$CHCl_3$. This compound decomposed on attempted vacuum distillation.

idates **5–19** were prepared using the general method developed by Atherton, Openshaw, and Todd.[8] To an ice-cooled and well-stirred solution of Et_3N (0.1 mol) in 50 ml of CCl_4 under N_2 was added the appropriate phosphite diester (0.1 mol) in 75 ml of CCl_4. The solution was heated to reflux with a precipitate forming. A solution of the appropriate amine (0.1 mol) in 50 ml of CCl_4 (0.05 mol in 25 ml of $CHCl_3$ for **19**) was added dropwise and additional precipitation occurred. The reaction mixture was stirred at reflux and/or at room temperature for a total of 1.5–144 hr. The mixture was cooled and filtered, and the filtrate was extracted with 5% HCl (2 × 25 ml), 5% $NaHCO_3$ (2 × 25 ml), and H_2O (25 ml) and dried (K_2CO_3). Concentration *in vacuo* gave the crude phosphoramidates which were purified by recrystallization or distillation (**5–18**) or by chromatography (**19**). Details are given in Table I.

Assay for *in Vitro* Antiplaque Activity.[5] Sterilized extracted human teeth were immersed in 1,2-dimethoxyethane solutions of the test compounds for two 1-min periods, each of which was followed by a 1-min exposure to air. These treated teeth were washed with 250 ml of distilled water for 5 min and then incubated under anaerobic conditions (BBL-Gaspak, BBL, Division of Bioquest, Cockeyville, Md.) in a sucrose-containing trypticase broth with *Streptococcus mutans* No. 6715, a pure strain of plaque-forming bacteria (isolated at and made available to us by the National Institute of Dental Research). The teeth were suspended in test tubes on orthodonic wire (Rocky Mount, 0.71 mm in diameter) threaded through a hole in the root so that the entire tooth was completely immersed. After 24 and 48 hr subjective estimates were made of adherent microbial growth on the test tube walls, wires, and the teeth and of nonadherent growth in the broth using a scale from 0 (no growth) to 4 (maximum growth). The total microbial accumulation was considered as *in vitro* plaque. For our results, growth ratings of 0 or 1 have been scored as plaque inhibition. Each compound was evaluated on five teeth with per cent inhibition values being reported. This indicated the percentage of teeth which did not show plaque formation after the stated incubation period. The solvent served as the control.

Acknowledgment. The authors gratefully acknowledge the support of this work by the National Institute of Dental Research, Grant No. 1 R01 DE03212-01A1.

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Fibrin-Stabilizing Factor Inhibitors. 10. Thiol Esters as Potent Enzyme Inhibitors

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Received April 16, 1973

A series of thiol esters has been prepared and tested as inhibitors of the fibrin-stabilizing factor. Two of the compounds were found to be the most active inhibitors so far described in the literature.

Synthetic anticoagulants which selectively inhibit the fibrin-stabilizing factor (FSF, factor XIII) have not been clinically tried so far. However, because such inhibitors

Table I. Physical Data and Inhibitory Activities of the Thiol Esters

$$R_1COSCH_2CH_2\overset{+}{N}(R_2)(R_2)(R_3)\ X^-$$

Compd no.	R_1	R_2	R_2	X^-	Mp, °C	Yield, %	Formula	Min inhibitory concn ± 0.15 m*M*[a]
1	C_6H_5	C_2H_5	H	Cl	140–141[b]	79	$C_{13}H_{19}NO_3 \cdot HCl$	16.4
2	C_6H_5	C_2H_5	CH_3	I	118–119	48	$C_{14}H_{22}INOS$	19.0
3	C_6H_5	C_2H_5	$C_6H_5CH_2$	Br	141–143	26	$C_{20}H_{26}BrNOS$	8.3
4	*trans*-$C_6H_5CH{=}CH$	CH_3	H	Cl	179–180[c]	55	$C_{13}H_{17}NOS \cdot HCl$	>9[e]
5	*trans*-$C_6H_5CH{=}CH$	CH_3	CH_3	I	241–242	83	$C_{14}H_{20}INOS$	>9[e]
6	*trans*-$C_6H_5CH{=}CH$	CH_3	$C_6H_5CH_2$	Br	171–172	40	$C_{20}H_{24}BrNOS$	4.5
7	*trans*-$C_6H_5CH{=}CH$	C_2H_5	H	Cl	157–158[d]	72	$C_{15}H_{21}NOS \cdot HCl$	>9[e]
8	*trans*-$C_6H_5CH{=}CH$	C_2H_5	CH_3	I	146–148	49	$C_{16}H_{24}INOS$	12.2
9	*trans*-$C_6H_5CH{=}CH$	C_2H_5	$C_6H_5CH_2$	Br	163–165	60	$C_{22}H_{28}BrNOS$	4.0
10	$C_6H_5(CH_2)_2$	C_2H_5	H	Cl	84–86	73	$C_{15}H_{23}NOS \cdot HCl$	13
11	$C_6H_5(CH_2)_2$	C_2H_5	CH_3	I	51–53	20	$C_{16}H_{26}INOS$	7.3
12	$C_6H_5(CH_2)_2$	C_2H_5	$C_6H_5CH_2$	Br	111–112	56	$C_{22}H_{30}BrNOS$	3.0
13	$C_6H_5(CH_2)_3$	C_2H_5	H	Cl	92–93	57	$C_{16}H_{25}NOS \cdot HCl$	>9[e]
14	$C_6H_5(CH_2)_3$	C_2H_5	CH_3	I	57–59	63	$C_{17}H_{28}INOS$	9.8
15	$C_6H_5(CH_2)_3$	C_2H_5	$C_6H_5CH_2$	Br	*f*	80	$C_{23}H_{32}BrNOS$	3.5

[a] Assayed as described.[6] Minimum inhibitory concentration that to 100% inhibits the formation of the γ–γ dimer. [b] Reported[9] 137–138.5°. [c] Reported[9] 178.5–179.4°. [d] Reported 156.5–158°. [e] Insoluble in higher concentrations at the pH of the test system. [f] Gave an oil with correct C, H, and N analyses.

might have considerable value as thrombolytic aids,[1] we are currently trying to develop new inhibitors of FSF which could be potentially useful in combating thrombotic diseases.

The cross-linking of fibrin is a transamidation reaction producing intermolecular γ-glutamyl-ϵ-lysine bridges. In this process, the reacting glutamine residue can be regarded as an acceptor group and the lysine residue as a donor.[2] Similarly, inhibitors of FSF can function either as acceptors or donors. Inhibitors of the donor type have been extensively studied in recent years,[2,3] while very little progress has been made in the development of acceptor inhibitors.

It has been suggested[4] that the fibrinoligase-catalyzed cross-linking of fibrin proceeds *via* an acyl enzyme intermediate, *viz.* a thiol ester. This acceptor may then react with the ϵ-amino group of lysine (the donor) to form the intermolecular amide bond between the fibrin molecules. Consequently, it is possible that synthetic thiol esters could inhibit cross-linking by functioning as pseudo-acceptors.

Recently, Lorand, *et al.*,[5] reported that esters between thiocholine and a variety of carboxylic acids inhibited the enzymatic cross-linking of fibrin. We decided to further study this type of compounds, initially by synthesizing esters of 2-amino- or 2-ammonioethanethiols with methyl, ethyl, or benzyl groups at the nitrogen. The thiols were esterified with benzoic, cinnamic, 3-phenylpropionic, or 4-phenylbutyric acid. Thiocholine esters of these acids were also tested by Lorand, *et al.*[5] The thiol esters of phenylacetic acid were too unstable to be used as inhibitors.

The esters of simple alkyl mercaptans with benzoic acids or higher homologous acids are normally too insoluble in water to be tested as FSF inhibitors. However, esters of nitrogen-containing thiols, like thiocholine, have a much greater solubility and can therefore be assayed in comparatively high concentrations.

Chemistry. The esters were obtained by reacting the appropriate acid chloride and the 2-dialkylaminoethanethiol in benzene. The products were then quaternized using methyl iodide or benzyl bromide. The compounds prepared and their inhibitory activities are presented in Table I.

Bioassay. The bioassays were carried out using the gel electrophoretic method previously described.[6] Clots were formed either in the absence or presence of the inhibitors and then reduced with 2-mercaptoethanol. The proteins were separated using SDS-disk gel electrophoresis. In the absence of inhibitor three bands due to the α- and β-chain monomers and the γ–γ dimer can be observed on the gel. In the presence of inhibitors, the cross-linking is partially inhibited, and, in addition to the three bands mentioned, a band due to the γ-chain monomer can be seen. When the inhibition is complete, no band due to the γ–γ dimer can be observed on the gel.

This method can be used as an inhibitor assay by determining the minimum inhibitor concentration that prevents the formation of the γ–γ band. Monodansylcadaverine was used as an internal standard.

Results and Discussion

The activities of the thiol esters tested are collected in Table I. The results presented show that the structures of both the acyl and the thiol parts are of importance for the activity.

Comparing the activities of esters of the same thiol (*e.g.*, **2**, **8**, **11**, and **14** or **3**, **9**, **12**, and **15**), it is apparent that the activity increases from benzoyl (**2** and **3**) *via* cinnamoyl (**8** and **9**) to 3-phenylpropionyl (**11** and **12**) thiol ester. In both series, the 3-phenylpropionyl derivatives **11** and **12** are the most active inhibitors, whereas the activity decreases again for the next higher homologs **14** and **15**. The higher activity observed by increasing the number of carbons in the acyl part of the esters is thus not merely a function of the more lipophilic character of the higher homologs but also reflects a structural specificity of FSF for the acyl part of the molecules. A similar specificity has previously been established[7] for inhibitors of the donor type.

As shown in Table I, compounds with both tertiary and quaternary nitrogens have inhibitory activity. However,

the tertiary bases are in most cases too insoluble at the pH of the test solution (pH 7.4) to be studied in detail.

The lipophilic character of the thiol part of the esters appears to be of importance for the inhibitory activity. This can be observed by small structural changes such as the replacement of methyl with ethyl groups at the quaternary nitrogen (*cf.* **6** and **9**). A remarkable increase in activity is obtained by the introduction of a benzyl group as can be seen within each series of esters in Table I (*cf.* **2** and **3**; **8** and **9**; **11** and **12**; **14** and **15**). Generally, it may be said that the benzyldiethylammonium thiol esters **3**, **9**, **12**, and **15** are two or three times more active than the corresponding diethylmethylammonium thiol esters **2**, **8**, **11**, and **14**. It seems likely that this increased activity is due to the higher lipophilicity of the benzyl derivatives.

The most active inhibitor of FSF hitherto described in the literature is monodansylcadaverine. In the bioassay used in this study, monodansylcadaverine can inhibit the cross-linking at a minimum concentration of 4.0 m*M*. Two of the thiol esters, **6** and **9**, in Table I have approximately the same inhibitory activity as monodansylcadaverine, whereas two other esters, *viz.* 2-benzyldiethylammonioethyl 3-phenylthiolpropionate (**12**) and 2-benzyldiethylammonioethyl 4-phenylthiolbutyrate bromide (**15**) can inhibit the cross-linking at concentrations of only 3.0 and 3.5 m*M*, respectively. These are the most active inhibitors of the fibrin-stabilizing factor so far described.

Experimental Section

General Comments. Melting points were determined with calibrated Anschütz thermometers in an electrically heated metal block. All crystallized compounds were characterized by elemental analyses (C, H, N), which were within ±0.4% of the theoretical values, and ir spectra, which were run for identification purposes on a Perkin-Elmer 237 spectrophotometer.

Synthesis. The thiol esters carrying a tertiary amino group, **1**, **4**, **7**, **10**, and **13**, were all prepared as described for **1**.[8] These were then quaternized using methyl iodide or benzyl bromide as described for **2**, yielding **2**, **3**, **5**, **6**, **8**, **9**, **11**, **12**, **14**, and **15**.

2-Diethylaminoethyl Thiolbenzoate (1). Benzoyl chloride (4.7 g, 33.4 mmol) in dry benzene (50 ml) was slowly added at room temperature to a solution of 2-diethylaminoethanethiol (4.7 g, 35.1 mmol) and triethylamine (3.6 g, 35.4 mmol) in benzene (30 ml). After 3 hr the triethylamine hydrochloride was filtered off and the filtrate evaporated yielding the title compound as an oil (7.0 g, 88%). The hydrochloride had mp 140-141° (from EtOH) (reported[9] 137-138.5°).

2-Diethylmethylammonioethyl Thiolbenzoate Iodide (2). Compound **1** (1.3 g, 5.5 mmol) in dry acetone was allowed to react with methyl iodide (2.5 g, 17.6 mmol) at room temperature for 48 hr. A crystalline compound (48%) was obtained, mp 118-119° (from EtOH). *Anal.* ($C_{14}H_{22}INOS$) C, H, N.

Acknowledgments. We wish to thank professor Laszlo Lorand, Northwestern University, and Dr. Ragnar Lundén, AB Kabi, Stockholm, for valuable advice and discussions. This work has been supported by grants from the Swedish Medical Research Council (Project No. K72-13X-3770-01) and from AB Kabi, Stockholm, Sweden. The 2-diethylaminoethanethiol used was a gift from Evan's Chemetics, Inc., Darien, Conn.

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Three New Adenosine Triphosphate Analogs. Synthesis and Effects on Isolated Gut

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Received May 15, 1973

ATP is known to induce relaxation of isolated mammalian gut preparations,[1-6] and evidence has been presented indicating that a purine nucleotide, probably ATP, is the transmitter released from nonadrenergic, noncholinergic inhibitory nerves to the gastrointestinal tract.[5-9] The pharmacological responses of ADP, AMP, and adenosine are substantially modified by purine ring or phosphate chain substitutions. For example, the platelet-aggregating properties of ADP are greatly enhanced by substitution of chloro or methylthio groups in position 2 of the purine ring and are abolished when the anhydride oxygen of the pyrophosphate moiety is replaced by a methylene group.[10] 2-Methylthio-AMP is a significantly more potent inhibitor of ADP-mediated platelet clumping than is AMP[11] and 2-chloroadenosine has more potent and longer lasting coronary and peripheral vasodilator effects than adenosine.[12] Mihich, *et al.*,[2] reported that 2-chloroadenosine was 2–3 times more active than adenosine in inhibiting spontaneous motility and tone in the isolated rabbit intestine. In order to compare the effects of analogous structurally modified derivatives of ATP on the isolated gut with those of ATP, we have synthesized 2-chloroadenosine 5′-triphosphate (2-chloro-ATP), 2-methylthioadenosine 5′-triphosphate (2-methylthio-ATP), and 2-chloro-5′-adenylyl methylenediphosphonate (2-chloro-AOPOPCP). We compared the relaxations elicited by these analogs and by ATP on the guinea-pig isolated taenia coli.

2-Chloro-ATP and 2-methylthio-ATP were synthesized in good yield by the general method of Moffat[13] from the 4-morpholine-*N*,*N*′-dicyclohexylcarboxamidinium salts of the appropriate adenosine 5′-phosphoromorpholidate analogs, the syntheses and characterization of which have been described elsewhere.[10,14] Preparations of 2-chloroadenosine 5′-phosphoromorpholidate contained 2-morpholinoadenosine 5′-phosphoromorpholidate as a minor contaminant, as previously noted,[10] resulting from nucleophilic substitution of chlorine by morpholine at the 2 position. Thus, although 2-chloroadenosine 5′-phosphoromorpholidate was chromatographically homogeneous in two different solvent systems and gave a satisfactory elemental analysis, it exhibited a bright blue fluorescence under ultraviolet light, in contrast to both 2-chloro-AMP and the 4-morpholine dicyclohexylcarboxamidinium ion; it also possessed an anomalous uv spectrum at pH 1, with a small extra peak at 300 nm. Replacement of 2-chloro-AMP by 2-chloroadenosine in the phosphoromorpholidate synthesis[10,14] resulted in the formation of a by-product, which was isolated in 45% yield after separation from unchanged starting material and characterized as the previously unreported nucleoside 2-morpholinoadenosine. Whenever the 2-chlo-