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Biochemically Active Substances from Microorganisms. V.[1] Pyrrothines, Potent Platelet Aggregation Inhibitors of Microbial Origin

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Pyrrothines, known as broad-spectrum antibiotics, showed potent membrane stabilization and platelet anti-aggregant activities. Thiolutin, the most potent among them, is three times as active as indomethacin in HH, APA and CPA, but has no activity in the PD system. Pyrrothines exhibited strong inhibitory activity on 5-HT release from bovine platelets, which may be responsible in part for their platelet anti-aggregant activity.

Keywords——pyrrothines; thiolutin; aureothricin; isobutyropyrrothine; membrane stabilizer; 5-HT release; platelet aggregation

As described in our previous paper,[1] we have developed *in vitro* screening systems applicable to microbial culture broths. These probes[3] were considered to be suitable for primary screening for new pharmacologically active compounds, especially anti-inflammatory compounds.

During the course of screening microbial culture broths, a streptomycetes, *Streptoverticillium* sp. AR10–AV10HB, was found to show strong inhibition of rat erythrocyte hemolysis. The active principle involved in the stabilization of the rat erythrocyte membrane has been purified and identified as three homologous pigments of the pyrrothine group of antibiotics, which also showed strong inhibition of APA and CPA, but not PD. This is the first report of a potent platelet anti-aggregant of microbial origin. In the present paper, we describe the activity of pyrrothine antibiotics elucidated using these *in vitro* probes, and the mode of action of the antibiotics on bovine platelets.

Experimental

Inhibition of Bovine Platelet Aggregation (APA and CPA)——The reversible aggregation induced by ADP (APA) and the irreversible aggregation induced by soluble collagen (CPA) were assayed by the methods

1) Part IV: Y.T. Ninomiya, Y. Yamada, M. Onitsuka (née Ono), Y. Tanaka, T. Maeda, and H.B. Maruyama, "Biochemically Active Substances from Microorganisms. IV. Establishment of Several *In Vitro* Anti-inflammatory Probes Applicable for Microbial Broth and Effect of Non-steroidal Anti-inflammatory Drugs and Antibiotics on Them" *Chem. Pharm. Bull.*, **28**, 2553 (1980).

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3) The established systems were (*a*) primary platelet aggregation by ADP (APA), (*b*) secondary platelet aggregation by soluble collagen (CPA), (*c*) rat erythrocyte heat hemolysis (HH), and (*d*) protein heat denaturation (PD).

described in the preceding paper,[1] using partially purified bovine platelets.

Heat Hemolysis of Rat Erythrocytes (HH)——The assay was done by the method described previously.[1] Briefly, a one percent (w/v) suspension of freshly prepared rat erythrocytes in 0.15 M phosphate buffer, pH 7.4, was heated with or without test samples at 54° for 25 min and the hemolysis was determined spectrophotometrically at A_{540} after centrifugation.

Inhibition of Protein Denaturation (PD)——Heat denaturation of 0.2% (w/v) bovine serum albumin (Fraction V, Sigma) in 0.2 M phosphate buffer, pH 5.3, was assayed by the method described previously,[1] in which the solution was heated at 67° for 4.5 min in the presence or absence of the test samples, and the turbidity at 650 nm was followed.

Pyrrothine Producer Strain and Culture Conditions——The pyrrothine-producing strain, *Streptoverticillium* sp. AR10-AV10HB, was isolated from an Australian soil sample collected in Darwin. The fermentation was done on a flask scale (500 ml Erlenmeyer flask containing 100 ml of D medium) with shaking at 180 strokes/min at 27° and the broth collected at day 4 was used in the isolation studies. D medium contains 1% glucose, 1% soluble starch, 1.5% soybean meal, 0.3% NaCl, 0.1% K_2HPO_4, 0.1% $MgSO_4$–$7H_2O$, and 1 ml/l of metal mixture ($MnCl_2$ 0.008%, $CuSO_4$ 0.007%, $ZnSO_4$ 0.002%, and $FeSO_4$ 0.0001%).

Isolation and Identification of Pyrrothines——The cultivated broth (5 l) was filtered and the filtrate was extracted twice with *n*-butyl acetate (1st, 3 l; 2nd, 2 l). The extracts were concentrated under reduced pressure to give a yellowish powder (IC_{50}; 10 μg/ml in HH). Silica gel thin-layer chromatography with chloroform-methanol (30 : 1) indicated the presence of at least three inhibitors, I (*Rf* 0.44), II (*Rf* 0.56) and III (*Rf* 0.64), in the crude preparation.

The crude material was applied to a column of Wako-gel C-200 (31 × 390 mm) equilibrated with chloroform, which was then eluted with chloroform. The following active fractions were combined, and concentrated, and each residue was crystallized from chloroform–ethanol: Fractions No. 100—165, 220 mg of I as yellow needles; Fractions No. 68—89, 32 mg of II as yellowish-orange needles; Fractions No. 50—65, 16 mg of III as orange needles.

All these inhibitors exhibited almost identical UV absorption spectra (λ_{max}; 245, 290, 310, 388 nm), characteristic of pyrrothine antibiotics.[4-7] Mass spectra of I, II and III showed the highest peaks at *m/e* 228, 242 and 256, respectively. These data suggested that I, II and III were identical with known pyrrothine antibiotics, thiolutin (molecular weight; 228),[5] aureothricin (molecular weight; 242)[6] and isobutyropyrrothine (molecular weight; 256),[7] respectively. This was confirmed by comparison of their IR spectra, mass spectra, antimicrobial spectra and chromatographic behavior with those of authentic samples.

5-HT[8] Uptake or Release from Bovine Platelets——Freshly prepared bovine platelets were obtained by the method described in the preceding paper.[1] To 5×10^8 bovine platelets/ml preincubated with samples (drugs) at 37° for 20 min, 1 μM 5-HT (^{14}C) was added. Aliquots of the reaction mixture were taken after incubation for 1—40 min, centrifuged, and counted to determine 5-HT (^{14}C) uptake.

The release of 5-HT was measured with platelets preincubated with 1 μM 5-HT (^{14}C) (final concentration) at 37° for 60 min, by adding 20 μg/ml collagen and 5 mM $CaCl_2$ (final concentration) and incubating the mixture for 1—20 min with stirring. An aliquot of the reaction mixture was taken, chilled rapidly, and centrifuged, then the supernatant was counted to determine 5-HT (^{14}C) release.

Effect on Thromboxane Biosynthesis from Arachidonic Acid[9]——Fresh bovine platelets were sonicated and centrifuged at 10000 × g for 30 min, and the supernatant was used as crude enzyme. 0.2 μCi of [^{14}C]-arachidonic acid (1.1 μg/ml) was incubated with 2.5 mM glutathione, 2 mM tryptophan and 1 mg/ml hemoglobin in 50 mM Tris-HCl, pH 7.5. The crude enzyme obtained from 8×10^9 bovine platelets/ml was then added and the mixture was incubated at 37° for 6 min. The reaction was stopped by the addition of 2 N HCl to pH 3.0. The reaction mixture was extracted with ethyl acetate, developed on a TLC plate (the solvent system was $CHCl_3$: MeOH: CH_3COOH: H_2O = 90: 8: 1: 0.8), and a radiochromatogram was obtained.

Chemicals——The chemicals used here were imipramine-HCl (Tofranil®, Geigy), aspirin (Nakakita Yakuhin Co. Ltd.), papaverine (Wako Pure Chem. Co. Ltd.), 5-hydroxytryptamine creatinine sulfate (Wako Pure Chem. Co. Ltd.), 5-hydroxy-[2-^{14}C]-tryptamine creatinine sulfate (58 mCi/m mol; Radiochemical Centre, Amersham, England), arachidonic acid-[1-^{14}C] (55.5 mCi/m mol, 181 μCi/mg; Radiochemical Centre, Amersham, England), and indomethacin. Thromboxane B_2 was kindly provided by Dr. S. Murota (Tokyo Metropolitan Institute for the Aged).

4) H. Umezawa (ed.), "Index of Antibiotics from Actinomycetes," Univ. Tokyo Press, Tokyo, 1968.
5) H. Seneca, *Antibiotic. Chemother.*, **2**, 357 (1952); W.D. Celmer and I.A. Solomons, *J. Am. Chem. Soc.*, **77**, 2861 (1955).
6) H. Umezawa, K. Maeda, and H. Kosaka, *Jap. Med. J.*, **1**, 512 (1948).
7) D.S. Bhate, P.K. Hulyalker, and S.K. Nenon, *Experimentia*, **16**, 504 (1960).
8) 5-HT; 5-hydroxytryptamine.
9) U. Diczfalusy and S. Hammarström, *FEBS Letter*, **82**, 107 (1977).

Results

Inhibition of Heat Hemolysis and Platelet Aggregation by Pyrrothines

When actinomycetes metabolites were assayed for membrane stabilization activity in rat erythrocyte heat hemolysis (HH),[1] *Streptoverticillium* sp. AR10–AV10HB was found to produce potent HH inhibitors in the culture broths. Purification following this activity by the methods described in "Experimental" revealed that the active principle for erythrocyte membrane stabilization was a mixture of homologous antibiotics; thiolutin (I),[5] aureothricin (II),[6] and isobutyropyrrothine (III),[7] the structure of which are shown in Fig. 1a.

All the purified pyrrothine homologs had potent HH inhibitory activity showing a biphasic dose-response profile, as commonly seen with known non-steroidal anti-inflammatory drugs (Fig. 1b). The activity was strongest with thiolutin ($IC_{50}=7.3\times10^{-6}$ M), which is 3 times stronger than indomethacin,[1] and decreased with elongation of the side chain in the amine. Thus, isobutyropyrrothine was 4 and 2 times less active than thiolutin and aureothricin, respectively. On the other hand, none of the pyrrothines showed any inhibition of osmotic hemolysis (OH)[10] (Table I).

Table I. Comparison of Activities on *In Vitro* Anti-inflammatory Screening Probes

Name	IC_{50} (M)				
	PD	HH	OH	CPA	APA
Pyrrothines:					
Thiolutin	NE	7.3×10^{-6}	NE	1.7×10^{-7}	8.0×10^{-8}
Aureothricin	NE	1.2×10^{-5}	NE	1.7×10^{-7}	2.9×10^{-7}
Isobutyropyrrothine	NE	2.9×10^{-5}	NE	3.3×10^{-7}	4.7×10^{-7}
Indomethacin	2.5×10^{-5}	3.2×10^{-5}	—	1.1×10^{-7}	NE
Aspirin	Weak ($IC_{30}=10^{-2}$)	Weak (1.25×10^{-3})	—	1.9×10^{-7}	NE
Papaverine	—	—	—	8.0×10^{-6}	8.0×10^{-6}
Dipyridamole	NE	NE	—	1.3×10^{-4}	1.6×10^{-4}
Imipramine	NE	$IC_{30}=1.3\times10^{-4}$	—	4.4×10^{-5}	4.7×10^{-5}

NE: no effect, —: not examined.

Further study showed that all of these substances potently inhibited both reversible and irreversible aggregation of bovine platelets induced by ADP (APA) and soluble collagen (CPA). Figure 1 c and d show the dose-response curves, which again indicate differential potency among the 3 homologs in accordance with that in the HH system, though aureothricin was as active as thiolutin in CPA. The IC_{50} value at the 10^{-7} M level is the strongest among drugs so far tested.[1] On the other hand, the compounds showed no inhibitory activity at all in PD.[1] This result, together with the similar extents of inhibition in CPA and APA (Table I), suggests a distinct difference in mode of inhibition from typical non-steroidal anti-inflammatory drugs, as discussed in the preceding paper.[1] When these properties were plotted quadratically, the pattern obtained resembled none of the known classifications (see Fig. 1, VI in Ref. 1), suggesting a unique target site of these homologs.

Effect of Pyrrothines on the Uptake and Release of 5-Hydroxytryptamine (5-HT)

Pyrrothines were found to have stabilizing activity on rat erythrocyte membrane. So-called membrane-stabilizing platelet aggregation inhibitors such as imipramine are known to inhibit 5-HT uptake into the dense body of the platelet.[11] In order to define the target of

10) J.W. Westley, *J. Antibiot.* (Tokyo), **29**, 584 (1976).
11) P.M. Seeman, *Int. Rev. Neurobiol.*, **9**, 145 (1966).

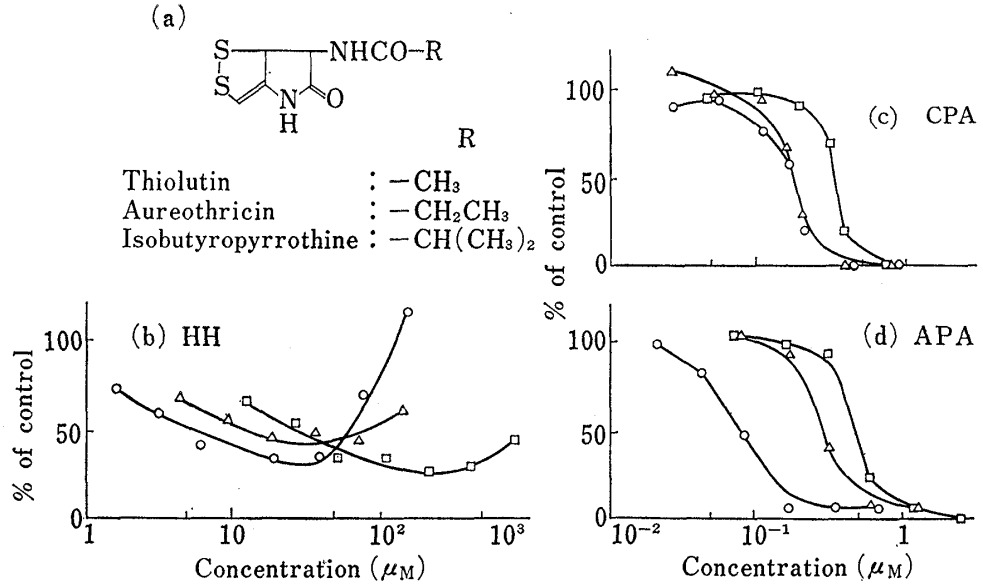

Fig. 1. Dose–Response Curve of Pyrrothines on (b) HH, (C) CPA, and (d) APA

—○—, thiolutin; —△—, aureothricin; —□—, isobutyropyrrothine.

pyrrothines on APA and CPA, the membrane-stabilizing activity on platelets was examined by means of 5-HT uptake experiments. As shown in Fig. 2a, 1 μg/ml of pyrrothines, aspirin, and indomethacin had no effect on the 5-HT uptake at all. The concentration of these tested samples was several times higher than their IC_{50} values in APA or CPA.

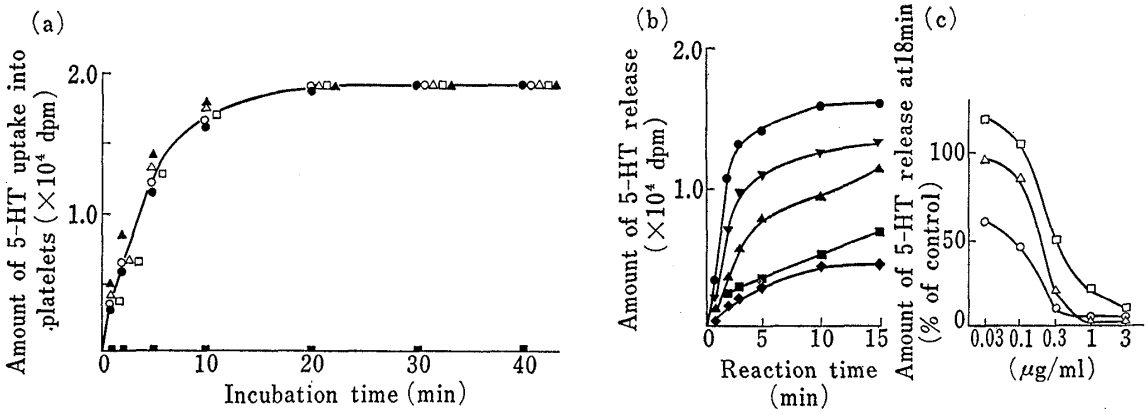

Fig. 2. 5-HT Uptake into Platelets (a), 5-HT Release from Platelets (b), and Dose-response Curve of Pyrrothines on 5-HT Release (c)

For conditions, see "Experimental".
—●—: control; —▲—: 1 μg/ml indomethacin; —■—: 50 μg/ml imipramine; —▼—: 1 μg/ml aspirin; —◆—: 10 μM papaverine; —○—: 1 μg/ml thiolutin; —△—: 1 μg/ml aureothricin; —□—: 1 μg/ml iso-butyropyrrothine.

In the case of CPA, 5-HT, ADP and other dense body components were released from platelets by activation with collagen, and then irreversible aggregation occurred. In order to check this stage, 5-HT release was examined. In contrast to the case of 5-HT uptake, thiolutin (1 μg/ml), aspirin (1 μg/ml), indomethacin (1 μg/ml), and papaverine (10 μM) inhibited 5-HT release (Fig. 2b).

IC_{50} values of pyrrothines against 5-HT release on incubation for 18 min were 0.44 μM for thiolutin, 0.78 μM for aureothricin, and 1.2 μM for isobutyropyrrothine (Fig. 2c). The order of activity was essentially the same as that obtained in the HH and APA systems.

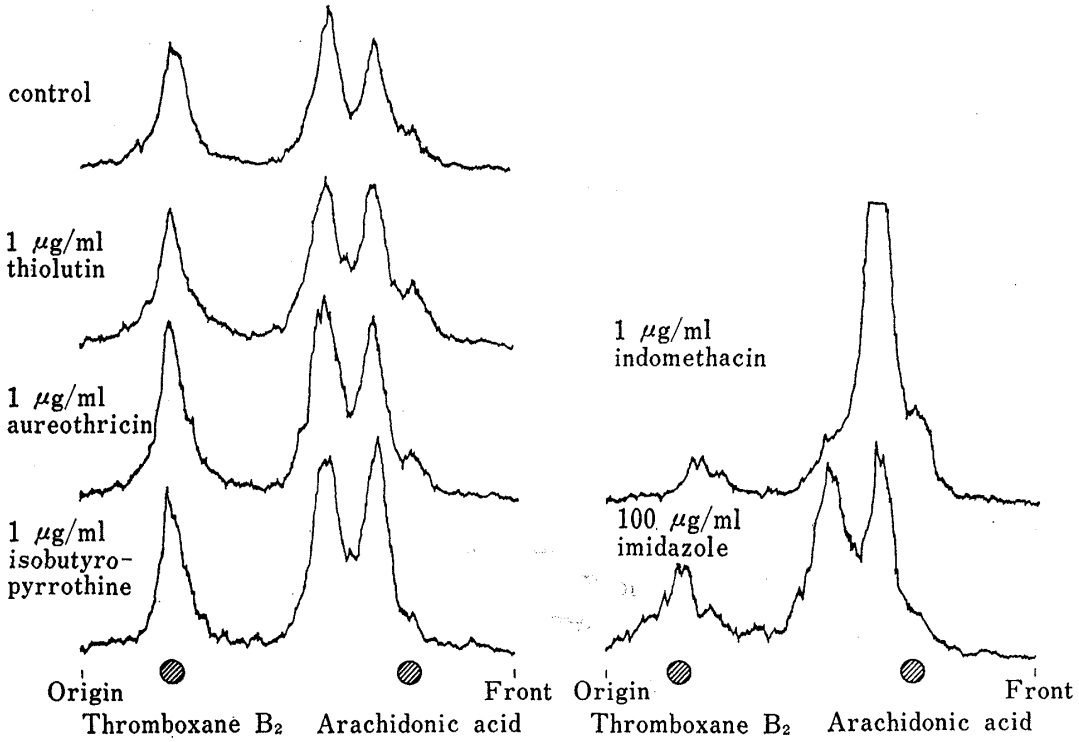

Fig. 3. Thromboxane Synthesis in Bovine Platelet Lysate

Conditions: lysate of 8.5×10^9 bovine platelets/ml; 1.1 μg/ml arachidonic acid (0.2 μCi); 2.5 mM glutathione; 2 mM tryptophan; 1 mg/ml hemoglobin; 50 mM Tris-HCl, pH 7.5; 37°, 6 min; solvent system, $CHCl_3$: MeOH: CH_3COOH: H_2O (90: 8: 1: 0.8).

Effect of Pyrrothines on Prostaglandin Biosynthesis

Non-steroidal anti-inflammatory drugs such as aspirin and indomethacin are known to inhibit prostaglandin biosynthesis, and hence irreversible platelet aggregation alone. As was expected from the finding that pyrrothines inhibited not only CPA but also APA, pyrrothines were found to have no effect on the biosynthesis of thromboxane B_2 from arachidonic acid, while indomethacin inhibited the biosynthesis of thromboxane B_2 (Fig. 3). Pyrrothines did not inhibit phospholipase A_2 activity in preliminary experiments (data not shown).

Discussion

Pyrrothines are broad-spectrum antibiotics found in 1952,[5] but their membrane stabilization activity and inhibition of platelet aggregation are new findings. The relative activity among the homologs was in the same order in the HH, APA and CPA systems: longer alkyl derivatives gave reduced inhibition in all three systems. They had no activity in the PD system.

The inhibitory activities of these pyrrothines on HH, APA and CPA are high. For example, thiolutin, the most active one, is three times as active as indomethacin on HH. Pyrrothines do not appear to be typical anti-inflammatory drugs like indomethacin, judging from patternal analysis of the differential effects on each *in vitro* probe,[1] since common non-steroidal anti-inflammatory drugs appear to have activities on HH, PD and CPA but not on APA. Actually they were shown to have no pharmacological effect on *in vivo* anti-inflammatory model systems, carrageenin edema and adjuvant arthritis (not shown).

Platelet aggregation inhibitors have been classified into three types: membrane stabilizers, prostaglandin biosynthesis inhibitors, and cyclic AMP enhancers. Non-steroidal anti-inflammatory drugs inhibit thromboxane biosynthesis from arachidonic acid, as well as the releasing step, and finally inhibit the irreversible aggregation of platelets. Pyrrothines inhibited the

5-HT releasing step, but had no effect on thromboxane biosynthesis. Non-steroidal anti-inflammatory drugs inhibit only CPA, while membrane stabilizers inhibit both APA and CPA. Pyrrothines inhibited both APA and CPA, and showed membrane stabilizing activity on rat erythrocyte heat hemolysis. On the other hand, pyrrothines did not inhibit 5-HT uptake into platelets. These results indicate that pyrrothines have a different mode of action on platelet aggregation from that of both non-steroidal anti-inflammatory drugs and typical membrane-stabilizing anti-aggregants.

Cyclic AMP enhancers, including phosphodiesterase inhibitors (for example, papaverine), inhibited CPA, APA and 5-HT release. Although the effect of pyrrothines on cyclic AMP level was not confirmed, preliminary experiments appeared to show an increase, and further studies are under way.

Thus, three possible modes of inhibition of platelet aggregation by pyrrothines may be considered at present: (1) increasing the cyclic AMP level in platelets, (2) inhibiting arachidonic acid release from membrane phospholipids through changes in the platelet membrane, or (3) acting *per se* as thromboxane antagonists. Further studies are required to identify the mechanism involved, but in any case the strong inhibitory activity on platelet aggregation and the rat erythrocyte membrane stabilization suggest that these compounds may also have unique pharmacological effects other than anti-inflammatory activity.

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