STUDIES ON NEW ANTIPLATELET AGENTS, WS-30581 A AND B

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WS-30581 A and B are antiplatelet agents produced by a strain of *Streptoverticillium waksmanii*. The compounds were purified by solvent extraction and column chromatography, and identified as new pimprinine analogs on the basis of their physico-chemical properties. The producing organism produced pimprinine along with these compounds. Pimprinine and the two new analogs have potent inhibitory effects on platelet aggregation.

There is current interest in the discovery of antithrombotic agents which block platelet thromboxane A_2 systhesis without interfering with vascular endothelium generation of prostacyclin also synthesized from arachidonic acid.

In our continuing search for potential antithrombotic agents, we have tested a wide range of fermented broths for inhibitory effects on platelet aggregation and thromboxane A_2 synthesis. As a result, active compounds were isolated from broth of strain No. 30581 of *Streptomyces* and identified as pimprinine (5,3′-indolylmethyloxazole) analogs by their physico-chemical data.

Pimprinine which exhibits antiepileptic[1] and monoamine oxidase inhibitory effects[2] was isolated by BHATA *et al.*[3] and synthesized by JOSHI *et al.*[4], but reports of its inhibitory effects on platelet aggregation and thromboxane A_2 synthesis have apparently not been published to date.

This report describes taxonomic studies on the producing strain, fermentation and isolation procedures, and physico-chemical and biological properties of tentatively designated WS-30581 compounds.

Methods

Fermentation

Seed flasks (500-ml), containing 100 ml of the seed medium consisting of soluble starch 3%, glucose 0.2%, cotton seed meal 1%, gluten meal 1%, dried yeast 1% and $CaCO_3$ 0.2% were inoculated with growth from a well-grown slant culture of *Streptomyces*, strain No. 30581. The flasks were shaken on a rotary shaker (220 rpm, 5.1 cm throw) for 3 days at 30°C. The content of the flasks was used to inoculate 160 liters of fermentation medium in a 200-liter stainless steel fermentor. The composition of the medium was as follows: soluble starch 3%, glucose 0.2%, cotton seed meal 1%, gluten meal 0.5%, dried yeast 0.5% and $CaCO_3$ 0.2%. The pH of the medium was adjusted to pH 7.0 before sterilization. Fermentation was allowed to proceed for 4 days at a temperature of 30°C, air flow of 160 liters per minute and with agitation of 250 rpm.

The active compounds present in the fermentation broth or in preparations obtained during the purification process were detected by their inhibitory activity against rabbit platelet aggregation and thromboxane A_2 synthesis of rat platelets.

Platelet Aggregation

Blood was collected from the central ear arteries of male Japanese White rabbits (2.5~3.0 kg body weight). The blood was prevented from coagulation with 1 volume of 3.8% sodium citrate to 9 volumes of blood. Platelet rich plasma (PRP) was prepared by centrifugation of the blood at 1,300 rpm for

10 minutes at 10°C. The PRP was diluted with platelet poor plasma obtained by further centrifugation of the blood at 3,000 rpm for 10 minutes. The platelet counts in the PRP used for aggregation studies were about 4.0×10^5 platelets/mm^3. Aggregometry was performed with platelet aggregating agents in a Sienco dual sample aggregometer (DP-247 E) at 37°C, using 0.3 ml combined volume of PRP and reagents in a cylindrical glass cuvette under constant stirring with a magnetic stirring bar. Platelet aggregation was measured turbidimetrically by recording changes in the light transmission of PRP during aggregation. Activities of inhibitors were expressed as IC_{50} *i.e.* concentrations required to inhibit the platelet aggregation responses by 50%. Collagen (Tokyo Kasei Co., Ltd.) was used in amounts (2~20 μg/ml for PRP) sufficient to induce a response that was 80 to 90% of the maximum obtainable. Arachidonic acid (Sigma) was used at a final concentration of 150 μM. Similarly a final concentration of ADP (Boehringer Mannheim), usually 1 to 5 μM was chosen to induce approximately 75% of the maximum aggregation.

Thromboxane A_2 Synthesis

Thromboxane A_2 (TXA_2) produced from reaction with rat platelets and arachidonic acid was bioassayed by contraction of rabbit mesentric artery. Rat blood was collected through the abdominal artery from ether anesthetized male Sprague-Dawley rats (200~250 g). PRP was prepared by the same method as that of rabbit PRP. The platelet counts in the PRP used for TXA_2 synthesis were about 5×10^5/mm^3. A reaction mixture consisted of 0.25 ml of rat PRP, 0.03 ml of arachidonic acid (500 μg/ml) and 0.02 ml of a test compound. The substrate (arachidonic acid) was added to start the reaction. After one minute reaction with constant mixing in aggregometer at 37°C, the mixture of prostaglandin endoperoxides and TXA_2 produced was measured by superfusion method using rabbit aorta as described previously[5]. Tyrode's solution contained indomethacin (1×10^{-6} g/ml) and a mixture of antagonists (atropine sulfate 10^{-7} g/ml, mepyramine maleate 10^{-7} g/ml, phenoxybenzamine hydrochloride 10^{-7} g/ml, cyproheptadine hydrochloride 10^{-6} g/ml, propranolol hydrochloride 2×10^{-6} g/ml). Changes of the tension were measured isometrically by means of force displacement transducers coupled to a polygraph. Activities of inhibitors were expressed as IC_{50}, *i.e.* concentration required to reduce the contraction responses induced by the reaction mixtures containing TXA_2 by 50%. In this method, IC_{50} of imidazole, which is known as a TXA_2 synthesis inhibitor, was 3 mM and coincided with that of imidazole measured by the *in vitro* system of TXA_2 synthesis from prostaglandin H_2[6].

Results and Discussion

Taxonomic Studies on Strain No. 30581

Strain No. 30581 was isolated from a soil sample obtained from Wajima City, Ishikawa Prefecture, Japan. The methods described by SHIRLING and GOTTLIEB[7] were employed for this taxonomic study. Morphological observations were made with light and electron microscopes from cultures grown at 30°C for 14 days on yeast extract - malt extract agar, oatmeal agar and inorganic salts - starch agar. The aerial mycelia of strain No. 30581 exhibit *Verticillati* (Plate 1) which is the characteristic of the genus *Streptoverticillium* Baldacci 1958. The mature sporophores formed *Rectiflexibiles* (Plate 1) with 10 to 20 spores in each chain. The spores were determined by electron microscopy to be cylindrical with rounded edges and measured $0.7 \sim 0.8 \times 1.5 \sim 1.8$ μm in size. Spore surfaces were smooth (Plate 2).

Cultural characteristics were observed on ten media described by SHIRLING and GOTTLIEB[7] and WAKSMAN[8]. Incubation was made at 30°C for 14 days. The color names used in this study were taken from the Color Standard (Nihon Shikisai Co., Ltd.). Aerial mass color was in the gray or red color series. No soluble pigment was produced. Results are shown in Table 1.

Cell wall analysis was performed by the methods of BECKER *et al.*[9] and YAMAGUCHI[10]. Analysis of whole-cell hydrolysates showed the presence of LL-diaminopimelic acid. Accordingly, the cell wall of this strain is classified as type I.

Plate 1. Spore chains of strain No. 30581 from 14 days culture on inorganic salts - starch agar. (×300).

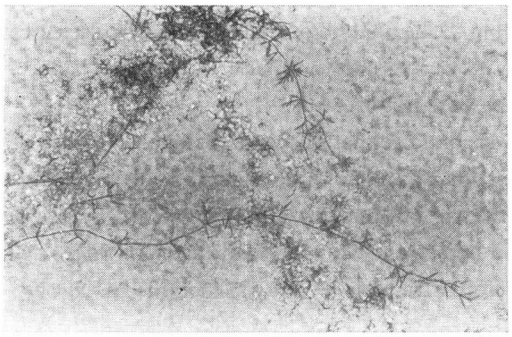


Plate 2. Electron micrograph of spores of strain No. 30581.
On inorganic salts - starch agar, 14 days (×7,200).

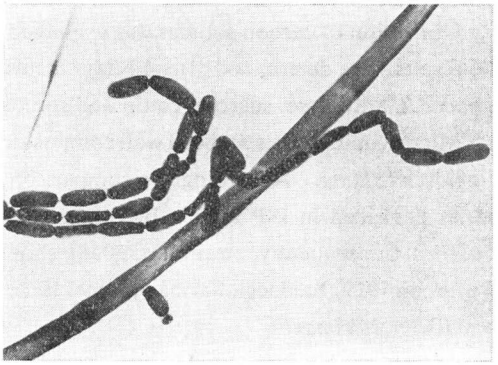


Table 1. Cultural characteristics of strain No. 30581.

Medium	Growth	Aerial mass color	Reverse side color	Soluble pigment
Oatmeal agar	Trace	None	Colorless	None
Yeast extract - malt extract agar	Abundant	Pinkish gray	Pale yellow orange	None
Inorganic salts - starch agar	Abundant	Pale yellowish gray	Pale greenish yellow	None
Glucose - asparagine agar	Moderate	Pale yellow green to grayish white	Yellowish brown	None
Sucrose - nitrate agar	Trace	None	Colorless	None
Glycerin - asparagine agar	Moderate	Grayish white	Yellowish brown	None
Nutrient agar	Moderate	None	Pale yellow	None
Potato - dextrose agar	Poor	Pale yellow green to grayish white	Yellowish brown	None
Tyrosine agar	Poor	None	Yellowish brown	None
Peptone - yeast extract - iron agar	Abundant	None	Yellowish brown	Brown

Table 2. Physiological properties of strain No. 30581.

Temperature range for growth	14~42°C
Optimum temperature	31~35°C
pH range for growth	3~9.5
Optimum pH	7
Nitrate reduction	+
Starch hydrolysis	+
Milk coagulation	−
Milk peptonization	±
Melanin production	+
Gelatin liquefaction	−
H_2S production	±
Urease activity	−

Symbols: + Positive, ± weakly positive, − negative.

Table 3. Carbon sources utilization of strain No. 30581.

Glycerin	+	D-Galactose	+
D-Xylose	−	L-Arabinose	−
Sodium citrate	+	D-Glucose	+
Lactose	−	Mannitol	+
D-Fructose	+	D-Mannose	+
Rhamnose	±	Sucrose	−
Maltose	+	Cellulose	−
Sodium succinate	+	D-Trehalose	+
Inulin	±	Salicin	±
Inositol	+	Chitin	−
Raffinose	−	Sodium acetate	−

Symbols: + Utilization, ± doubtful utilization, − no utilization.

Physiological properties of strain No. 30581 are shown in Table 2. Temperature range and optimum temperature for growth were determined on yeast extract - malt extract agar after 10 days incubation. Temperature range for growth was from 14°C to 42°C with optimum temperature from

31°C to 35°C. Starch hydrolysis and nitrate reduction were positive. Melanin production was positive on peptone - yeast extract - iron agar and Tryptone - yeast extract broth. On tyrosine agar, no melanoid pigment was produced.

Utilization of carbon sources was examined according to the method of PRIDHAM and GOTTLIEB[11]. The results were determined after 14 days incubation at 30°C. As shown in Table 3, D-xylose, lactose, raffinose, L-arabinose, sucrose, chitin and sodium acetate were not utilized.

Microscopic studies and cell wall composition analysis indicate that this strain belongs to the genus *Streptoverticillium*. Accordingly, a comparison of this strain was made with various *Streptoverticillium* species described in ISP reports by SHIRLING and GOTTLIEB[12~14] and the report described by LOCCI *et al.*[15]. Consequently, strain No. 30581 showed good agreement with *Streptoverticillium waksmanii* (Waksman 1919) Baldacci, Farina et Locci 1966. Therefore, strain No. 30581 was identified as *Streptoverticillium waksmanii*.

Isolation and Characterization of WS-30581 A and B

Fermentation broth (160 liters) was filtered with the aid of filter aid (Radiolite) and the mycelia were extracted with acetone. After evaporating acetone from the extract, the resulting aqueous residue was extracted with ethyl acetate at pH 7.0 and the extract was evaporated to dryness *in vacuo*. The oily material was applied to a column of silica gel. The column was washed with *n*-hexane and then eluted with a mixture of *n*-hexane and ethyl acetate (1 : 1). The active fractions were combined and concentrated to dryness. The dry residue was adsorbed on a reverse phase NS gel column (Nihon Seimitsu Kagaku Co., Ltd.). The column was eluted with methanol. The methanol effluent was concentrated *in vacuo*, and crystallized from benzene - *n*-hexane (1 : 1) yielding 1.5 g of a mixture of three components (WS-30581 A, B and C).

Preparative high performance liquid chromatography of this crystalline material (140 mg) on a Waters Associates, μBondapak C-18 column (mobile phase: 50% aqueous methanol) gave WS-30581 A (27 mg), B (14 mg) and C (5 mg). The physico-chemical properties of these compounds are summarized in Table 4.

Table 4. Physico-chemical properties of WS-30581 A, B and C.

	WS-30581 A	WS-30581 B	Pimprinine (WS-30581 C)
Appearance	Colorless needles	Colorless needles	Colorless needles
MP (°C)	128~130	123~125	204~205
Molecular formula	$C_{14}H_{14}N_2O$	$C_{15}H_{16}N_2O$	$C_{12}H_{10}N_2O$
MW (MS)	226	240	198
Anal Found	C 74.45, H 6.35, N 12.40	C 74.82, H 6.59, N 11.65	C 72.60, H 5.28, N 14.07
Calcd	C 74.31, H 6.24, N 12.38	C 74.97, H 6.71, N 11.66	C 72.71, H 5.09, N 14.13
IR (Nujol) cm^{-1}	3160, 1634, 1617, 1570, 1125, 1013, 1000, 918, 750, 745	3125, 1630, 1612, 1570, 1120, 1009, 920, 912, 775, 739	3140, 1635, 1615, 1595, 1125, 1024, 1016, 918, 778, 737
NMR ($CDCl_3$) δ	1.02 (3H, t, J=7 Hz), 1.87 (2H, m), 2.82 (2H, t, J=7 Hz), 7.10~7.50 (5H, m), 7.83 (1H, m), 9.58 (1H, br s)	0.96 (3H, t, J=7 Hz), 1.45 (2H, m), 1.80 (2H, m), 2.84 (2H, t, J=7 Hz), 7.10~7.92 (6H, m), 9.10 (1H, br s)	2.54 (3H, s), 7.10~7.98 (6H, m), 8.40 (1H, br s)
UV λ_{max}^{EtOH} (ε)	224 (24,000), 266 (15,400), 282 (sh 13,000), 298 (sh 11,200)	224 (22,200), 266 (14,400), 282 (sh 12,200), 298 (sh 10,400)	224 (24,600), 266 (15,500), 282 (sh 12,100), 298 (sh 11,000)

Fig. 1. IR spectrum of WS-30581 A (Nujol).

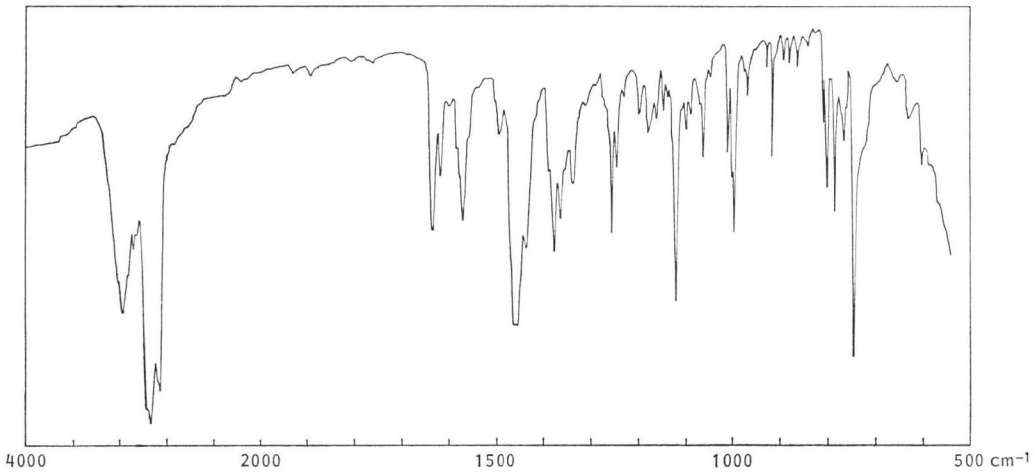
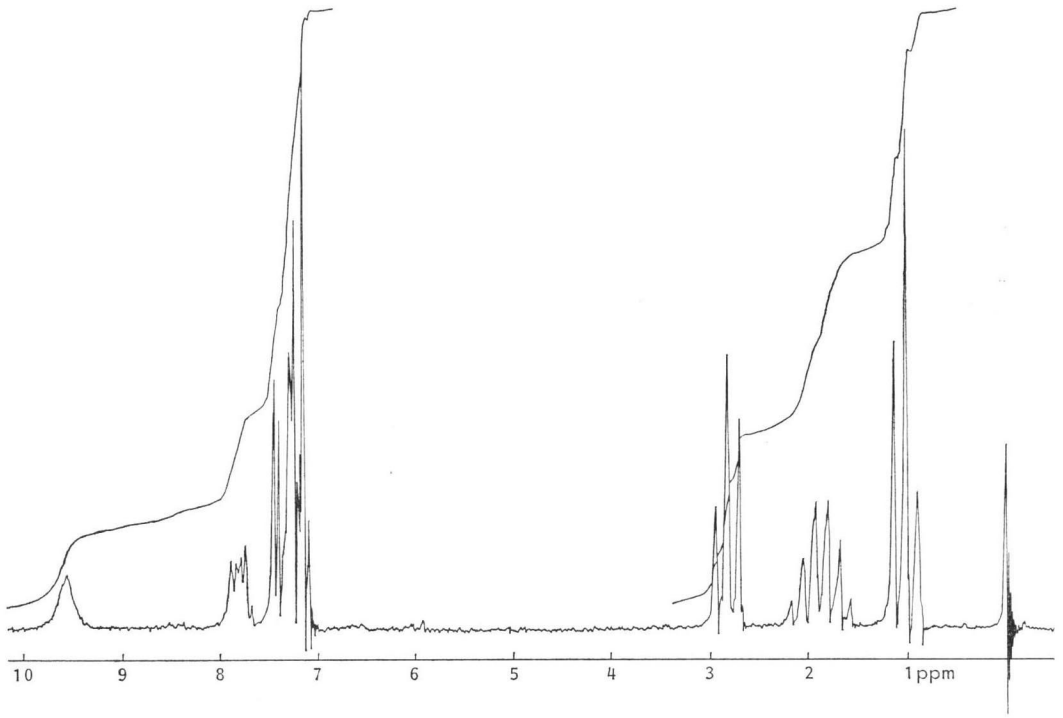
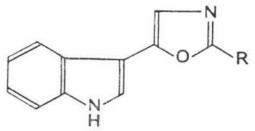


Fig. 2. ^{1}H NMR spectrum of WS-30581 A ($CDCl_3$).

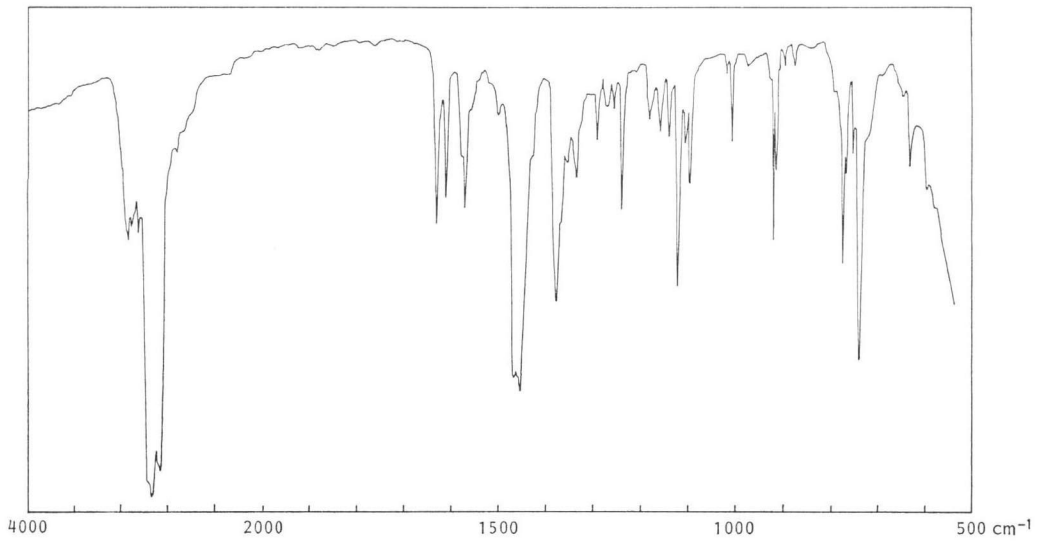


The three compounds were positive to Ehrlich test indicating the presence of the indole moiety. The spectral data resemble one another. WS-30581 C was established as pimprinine (**3**) by its physicochemical properties[4,16] and by synthesis according to the procedure of YONEMITSU *et al.*[17]. The major component WS-30581 A (**1**) has the molecular formula $C_{14}H_{14}N_2O$, established by elemental analysis and by mass spectrum. The IR (Fig. 1) and UV (Table 4) spectra were very similar to those of pimprinine (**3**). The NMR spectrum (Fig. 2) of WS-30581 A exhibited the signals at δ 1.02 (3H, t, J=7 Hz),



1 R=$CH_2CH_2CH_3$
2 R=$CH_2CH_2CH_2CH_3$
3 R=CH_3

Fig. 3. IR spectrum of WS-30581 B (Nujol).



1.87 (2H, m), 2.82 (2H, t, J=7 Hz), 7.10~7.50 (5H, m), 7.83 (1H, m) and 9.58 (1H, br, disappeared with D_2O) suggesting the presence of *n*-propyl group instead of methyl one in pimprinine. This fact indicated that the *n*-propyl side chain attached to C-2 position on oxazoline ring.

Based on the above mentioned evidence, the structure of **1** is most plausible to WS-30581 A.

The minor component, WS-30581 B (**2**), has the molecular formula, $C_{15}H_{16}N_2O$. Its spectral characteristics (Figs. 3, 4) showed similarity to WS-30581 A (**1**) and to pimprinine (**3**). The mass and NMR spectra revealed the presence of *n*-butyl chain in the molecule. Therefore, WS-30581 B must have the structure **2**.

Biological Properties

WS-30581 components show no antibacterial activity at a concentration of 100 μg/ml by agar dilution method against *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Proteus vulgaris* and *Candida albicans*. Acute toxicity of WS-30581 A in *ddY* mice is above 250 mg/kg by intraperitoneal injection.

The inhibitory activities of platelet aggregation *in vitro* are shown in Table 5. It is evident from this Table that WS-30581 components are nearly as active as aspirin against collagen, but are about 10 times more active than aspirin against arachidonic acid: they did not inhibit ADP induced platelet aggregation. Table 6 shows that WS-30581 components inhibit thromboxane A_2 synthesis, and they are 40 to 80 times stronger than imidazole, which is known as an inhibitor of thromboxane A_2 synthesis. However, the fact that the concentrations required to inhibit thromboxane A_2 synthesis are almost identical to those needed for inhibition of arachidonic acid induced platelet aggregation suggests that the mode of action of these pimprinine like compounds in inhibiting platelet aggregation might be aspirin-like and involve

Fig. 4. ^1H NMR spectrum of WS-30581 B ($CDCl_3$).

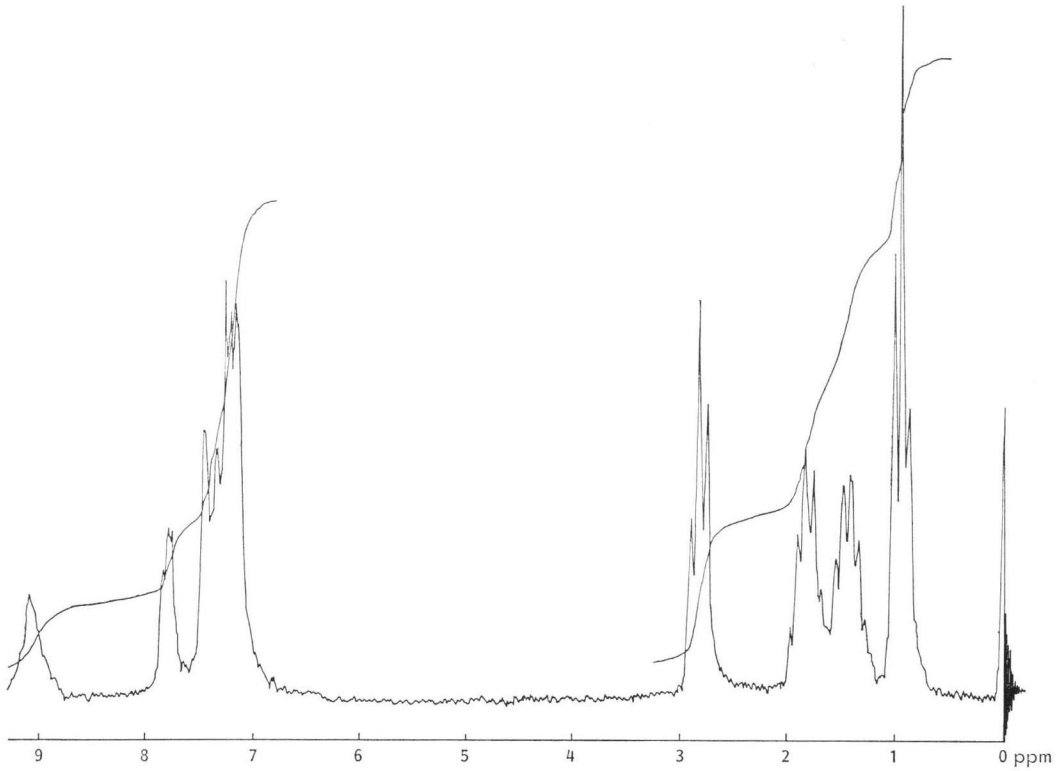


Table 5. Inhibition of rabbit platelet aggregation by WS-30581 A, B, C and aspirin.

Aggregating agent	IC_{50} (μg/ml)			
	A	B	C	Aspirin
Arachidonic acid	6	3	3	50
Collagen	25	25	25	25
ADP	>50	>50	>50	>50

Platelet aggregation was determined as described in the text. Results are presented as the concentration of each drug inhibiting maximal aggregation by 50%.

Table 6. Inhibition of thromboxane A_2 synthesis by WS-30581 A, B, C and imidazole.

Compound	IC_{50} (μg/ml)
WS-30581 A	3
B	3
C	6
Imidazole	240

Thromboxane A_2 synthesis was determined as described in the text. Results are presented as the concentration of each drug inhibiting maximal aggregation by 50%.

inhibition of thromboxane A_2 formation by irreversibly blocking platelet cyclooxygenase.

Pimprinine was first isolated by BHATA *et al.*[3] in 1960 as an inhibitory substance of convulsion caused by electric shock and mono-amine oxidase[2], but its inhibition of platelet aggregation is novel.

These compounds as described in this paper seem to be promising as antithrombotic agents, though further examination of antithrombotic activity in animal models is needed.

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