Sch 38519, A NOVEL PLATELET AGGREGATION INHIBITOR PRODUCED BY A *THERMOMONOSPORA* SP.

TAXONOMY, FERMENTATION, ISOLATION, PHYSICO-CHEMICAL PROPERTIES, STRUCTURE AND BIOLOGICAL PROPERTIES

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The complex containing a new platelet aggregation inhibitor, Sch 38519, was recovered from the fermentation filtrate of *Thermomonospora* sp. SCC 1793. A chemically defined medium was developed which favored the production of Sch 38519. The antibiotic was isolated from the fermentation filtrate by absorption on macroreticular resin and further purified by ion exchange chromatography and reverse phase HPLC. Sch 38519 is an isochromanequinone structurally related to medermycin, lactoquinomycin and granaticin. It inhibits thrombin-induced aggregation of human platelets with an IC_{50} of 68 μg/ml. Sch 38519 is also active against Gram-positive and Gram-negative bacteria.

In our screening program for unique microbial products with pharmacological activity, a fermentation filtrate from a *Thermomonospora* sp. exhibited platelet aggregation inhibitory activity. The major active component, Sch 38519, was purified and identified as a novel isochromanequinone[1], related to lactoquinomycin[2,3], medermycin[4~6] and granaticin[7,8]. The anti-platelet aggregation activity of this family of compounds has been recently reported[9]. This paper describes the taxonomy and fermentation of the producing culture, and the isolation, physico-chemical properties and biological activity of Sch 38519. Details of the structure elucidation and X-ray crystallographic analysis to determine the absolute stereochemistry are reported in a separate paper[10].

Taxonomy of the Producing Organism

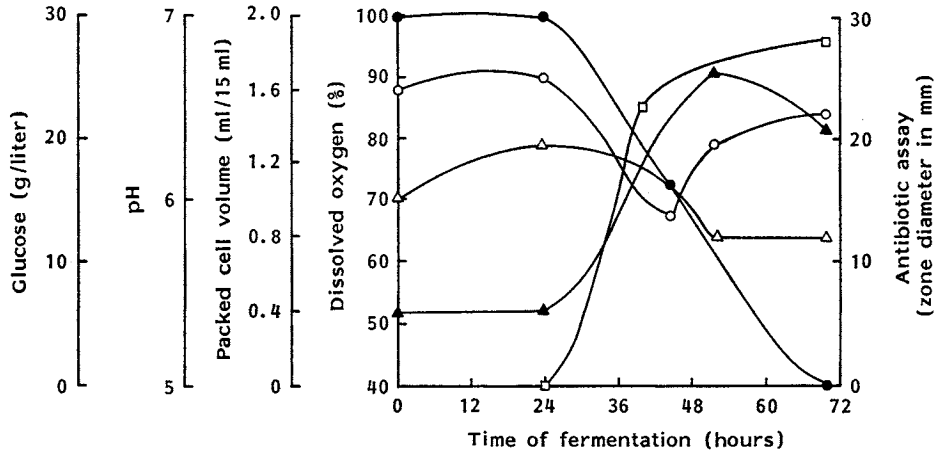
The producing culture, SCC 1793, was isolated from a soil sample collected in Wisconsin using standard soil dilution techniques and plating the dilutions on agar composed of yeast extract 0.1%, soluble starch 0.01%, agar 1.5% and everninomicin 10 μg/ml. Everninomicin was used as a selective agent to look for novel organisms.

SCC 1793 is filamentous, forming mycelia that differentiates into substrate mycelia that penetrate the agar appearing as a compact surface layer and aerial mycelia that originate from the substrate mycelia. On water agar, after 21 days at 30°C, abundant single spores are formed along the length and completely surrounding the branching aerial mycelia. The substrate mycelium is fine and branching, rarely forming single spores. After 21 to 28 days at 30°C, moderate to good growth occurs on the media of SHIRLING and GOTTLEIB[11], and WAKSMAN[12]. The substrate mycelia are yellow-brown to grayish-reddish orange. Yellow-brown to brown diffusible pigments are formed. The aerial mycelia, en masse, are white to pale yellowish-pink.

Whole cell analysis by the procedure of LECHEVALIER[13] indicates the presence of *meso*-diamino-

Fig. 1. Fermentation profile of antibiotic complex.

□ Antibiotic assay, ● dissolved oxygen, ▲ packed cell volume, △ pH, ○ glucose.



pimelic acid; diagnostic sugars are absent (Type C). Phosphatidylethanolamine and hydroxylated phosphatidylethanolamine are the characteristic phospholipids (Type PII).

Isolated spores are heat sensitive. The culture does not grow at 42°C; growth at 37°C is poor while good growth occurs at 20°C. Based on morphological and chemical analysis SCC 1793 is identified as a mesophilic species of *Thermomonospora*.

Fermentation

Stock cultures were maintained as frozen whole broths at −20°C in a final concentration of 10% glycerol. A 250-ml Erlenmeyer flask containing 70 ml of seed medium was inoculated with 7.0 ml of stock culture. The flask was incubated at 30°C on a rotary shaker at 300 rpm for 48 hours. The seed medium consisted of beef extract 0.3%, Tryptone 0.5%, yeast extract 0.5%, Cerelose 0.1%, potato starch 2.4%, $CaCO_3$ 0.2% and Dow-Corning antifoam B 1 ml per liter of tap water. The pH was adjusted to 6.4 before addition of $CaCO_3$. Fifty ml of this seed culture was used to inoculate a 2-liter Erlenmeyer flask containing 500 ml of the same seed medium and incubated as above. The entire contents of this second stage seed inoculum were used to inoculate a 14-liter fermenter containing 10 liters of a production medium consisting of yeast extract 1.75%, glucose 3.50%, Edamine 0.15%, Pharmamedia 0.15%, arabinose 0.50%, boric acid 0.00003%, EDTA 0.0001% in tap water. The pH was adjusted to 7.6 prior to sterilization. The fermentation was carried out for 72 hours at 28°C with an air flow of 3.5 liters per minute and an agitation rate of 350 rpm. A typical time course study in 14-liter fermenters is shown in Fig. 1. The production of Sch 38519 was monitored by measuring both platelet aggregation inhibition and antimicrobial activity against *Staphylococcus aureus* 209P.

Isolation

The steps leading to the isolation and purification of Sch 38519 are outlined in Fig. 2. After fermentation (30 liters), the cultured broth was filtered to remove the cells. The anti-platelet aggregation activity from the filtrate was absorbed on to Amberlite XAD-4 resin and the inactive spent filtrate decanted off. The charged resin was eluted with water - acetone. Further purification included ion-exchange on a cellulose based weak cation exchange CM-Sephadex C-25 (Na^+). Final purification of the major

Fig. 2. Isolation and purification of Sch 38519.

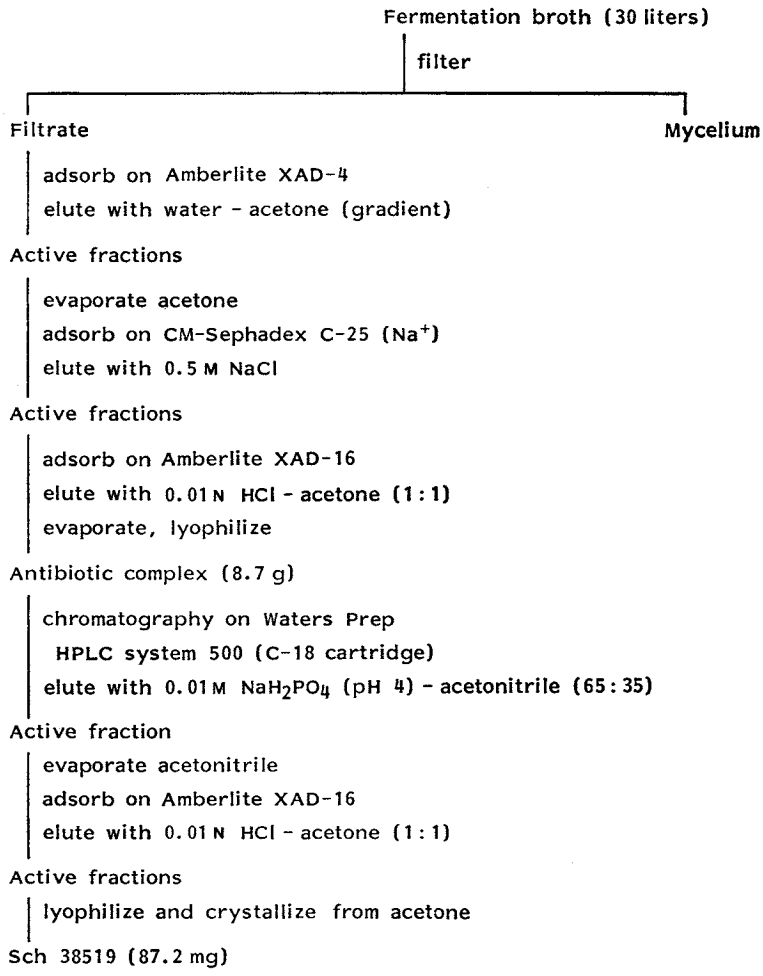


Table 1. Physico-chemical properties of Sch 38519.

Physical	Red needles, basic				
MP	215~220°C (dec)				
UV λ_{max} nm (ε)	217 (47,100), 266 (15,250), 438 (6,275)				
IR ν_{max}^{KBr} cm^{-1}	3420, 2935, 1790, 1670, 1650, 1620, 1450, 1410, 1310, 1220, 1150, 1050				
Analysis	Calcd for	C	H	N	Cl
	$C_{24}H_{26}NO_8Cl$:	58.60	5.32	2.89	7.21
	Found:	58.87	5.37	2.58	6.75
HRFAB-MS	Found 458.1854 (M+3H)				
	Calcd for $C_{24}H_{28}NO_8$ 458.1815				
$[\alpha]_D^{22}$	+74.5°				
CD $[\theta]$ nm	+56,000 (254), +81,000 (344), +118,000 (455), −86,000 (287)				

HRFAB-MS: High-resolution fast atom bombardment mass spectrum.

active compound was achieved by preparative HPLC on a reverse phase C-18 column eluting with 0.01 M phosphate buffer (pH 4) and acetonitrile (65:35). The major component between 18 and 30 minutes was collected. After desalting (Amberlite XAD-16 column), and upon crystallization from acetone gave bright red needles of Sch 38519 hydrochloride salt (87.2 mg).

Physico-chemical Properties

The hydrochloride salt of Sch 38519 is soluble in water and lower alcohols. It is stable in acidic solutions but rapidly decomposes above pH 8.0. The physico-chemical properties of this compound are shown in Table 1. The UV and IR spectrum are shown in Figs. 3 and 4. The determination of structure and absolute stereochemistry by spectral data and X-ray crystallographic analysis are reported in a separate paper[10]. The structure with absolute stereochemistry is shown in Fig. 5.

Biological Properties

Anti-platelet Aggregation Activity

Sch 38519 and lactoquinomycin A (obtained from University of Tokyo) were tested for inhibition of thrombin-induced aggregation and serotonin secretion in human platelets using the following assay procedures.

Platelet Aggregation: Platelet-rich plasma was prepared from freshly drawn human blood as previously described[14]. Sch 38519 and lactoquinomycin A were prepared as 10× stock solutions in Ca^{2+}- and Mg^{2+}-free phosphate buffered saline. Platelets were preincubated for 10 minutes at 37°C with the varying concentration of Sch 38519 and lactoquinomycin A. Human thrombin was then added to a final concentration of 1 U/ml and aggregation was monitored by the increase in light transmission on a Chrono-log aggregometer. Percent of maximal aggregation

Fig. 3. UV spectrum of Sch 38519.

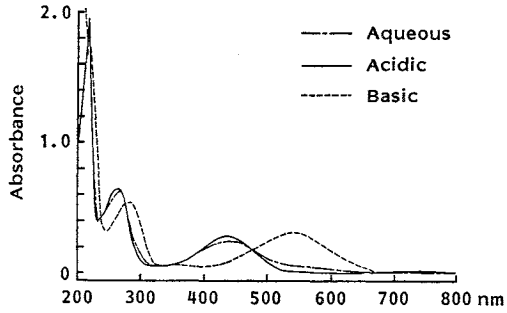


Fig. 4. IR spectrum of Sch 38519.

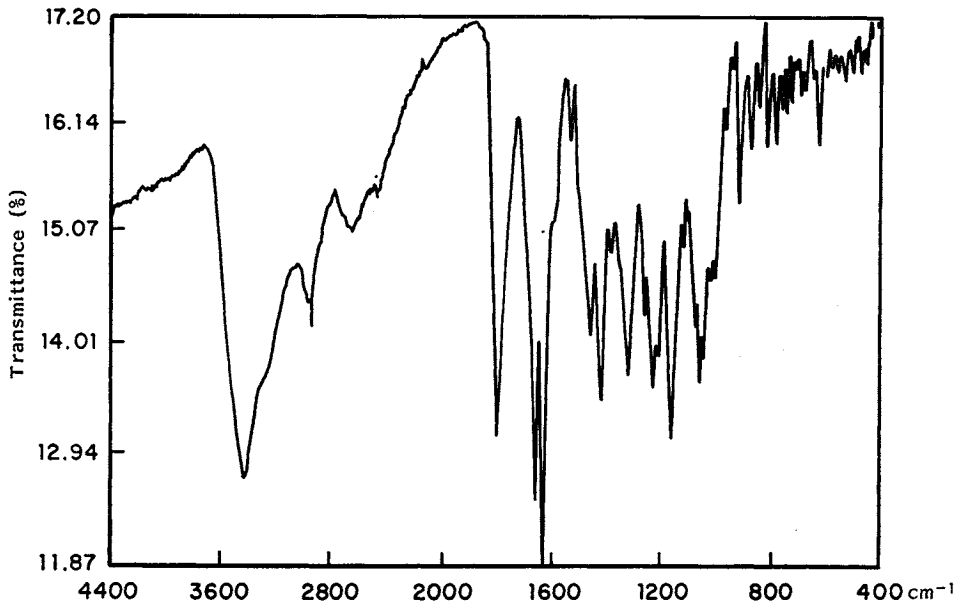
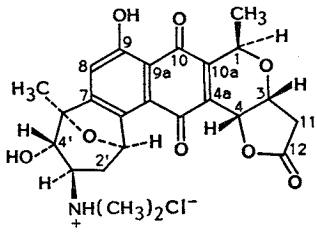


Fig. 5. Structure of Sch 38519 hydrochloride.



Sch 38519 (**1**)
(absolute configuration)

Table 2. *In vivo* activity of Sch 38519.

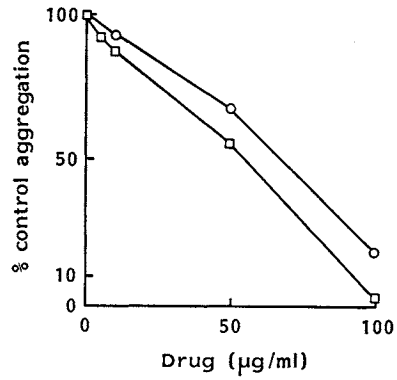
Organisms	No. of strains	Geometric mean MICs (μg/ml)[b]
Gram-negatives[a]	45	122.1
Gram-positives		
Staphylococcus	22	0.92
Streptococcus	32	0.82

[a] *Escherichia* (13), *Enterobacter* (4), *Klebsiella* (8), *Morganella* (1), *Providencia* (4), *Pseudomonas* (4), *Salmonella* (5), *Serratia* (5) and *Shigella* (1).
[b] 25 hours Mueller-Hinton agar, pH 7.4.

was calculated relative to the extent of the change in light transmission induced by thrombin in the absence of drug.

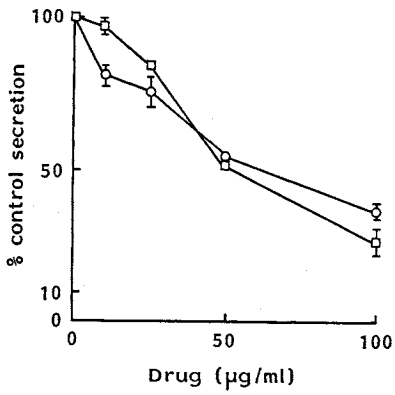
Sch 38519 and lactoquinomycin A inhibited both aggregation of thrombin-stimulated human platelet. IC_{50} values for inhibition of aggregation were 55 μg/ml for lactoquinomycin A and 68 μg/ml for Sch 38519 (Fig. 6). No effect of these compounds on the initial platelet shape change in response to thrombin was observed. The IC_{50} values observed for lactoquinomycin A and Sch 38519 is approximately 10-fold higher than that reported for the inhibition of rabbit platelet aggregation by medermycin[9].

Fig. 6. Inhibition of thrombin-induced platelet aggregation by lactoquinomycin A and Sch 38519.



Aggregation of human platelets was measured following pretreatment with the indicated concentration of lactoquinomycin A (□) or Sch 38519 (○). Data are presented as the percent of aggregation observed in the absence of drug.

Fig. 7. Inhibition of platelet serotonin secretion by lactoquinomycin A and Sch 38519.



Serotonin secretion was measured following pretreatment with the indicated concentration of lactoquinomycin A (□) or Sch 38519 (○). Data are presented as the percent of secretion observed in the absence of drug.

Serotonin Secretion: For measurement of serotonin secretion, platelet-rich plasma was incubated with 10 μCi/15 ml of [^{3}H]serotonin (New England Nuclear) for 45 minutes at 37°C. Platelets were then isolated as described by SIESS *et al.*[15] and resuspended to a concentration of 2.5×10^8 platelets/ml in TYRODE's buffer. Platelets were preincubated for 10 minutes with the indicated concentration of Sch 38519 or lactoquinomycin A (added from a 100× stock solution in Ca^{2+}- and Mg^{2+}-free phosphate buffered saline). [^{3}H]Serotonin secretion was then measured one minute after the addition of thrombin (1 U/ml final concentration) essentially as described by TOHMATSU *et al.*[16]. Data are expressed as percent of secretion observed in the absence of drug.

Both Sch 38519 and lactoquinomycin A inhibited secretion of [^{3}H]serotonin over the same concentration range as observed for inhibition of aggregation (IC_{50} values of 61 μg/ml for Sch 38519 and 53 μg/ml for lactoquinomycin A (Fig. 7)).

These data indicate that these isochromanequinone antibiotics can prevent the major physiological responses of platelets (aggregation and secretion) to a maximal stimulatory dose of thrombin.

The acute toxicity (iv, LD_{50}) of Sch 38519 in mice was 11 mg/kg. Sch 38519 displayed inhibitory activity against bacteria, particularly Gram-positive organisms, but no significant activity against fungi. The *in vitro* MICs against various Gram-negative and Gram-positive bacteria are presented in Table 2.

Discussion

The quest for obtaining unique natural products which can serve as therapeutic agents to treat a variety of diseases or serve as structural models to obtain a compound with favorable pharmacological activity and low toxicity is intense[17,18]. In this paper we report on a novel isochromanequinone possessing anti-platelet activity. A similar finding that isochromanequinones possess potent anti-platelet activity has been reported recently[9]. Sch 38519 and lactoquinomycin A show comparable IC_{50} values and appear 10-fold less active than medermycin based on reported data for medermycin[9]. Various classes of natural products have been used as inhibitors of platelet aggregation[19~21] but this represents a new class which is yet to be explored.

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