SULFOMYCINS, A SERIES OF NEW SULFUR-CONTAINING ANTIBIOTICS. I

ISOLATION, PURIFICATION AND PROPERTIES

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Isolation of a series of antibiotics produced by a new variety of *Streptomyces viridochromogenes* was attempted, and three main components were obtained as amorphous powders. They were primarily active against Gram-positive bacteria, anaerobic bacteria and mycoplasma and characterized as new members of sulfur-containing peptidic antibiotics. The antibiotic complex was named sulfomycin.

In the course of a screening program for antibiotics, a new variety of *Streptomyces viridochromogenes* (strain No. MCRL-0368) was found to produce a series of antibiotics primarily active against Gram-positive bacteria. The antibiotics with similar properties to each other were characterized as new members of sulfur-containing peptidic antibiotics, and named sulfomycin.

The present paper deals with isolation, purification, physicochemical and biological properties of three main components, sulfomycin I, II and III. Taxonomic studies on the sulfomycin-producing strain named *Streptomyces viridochromogenes* var. *sulfomycini* nov. var. will be reported later.

Isolation and Purification

During the isolation and purification process, antibacterial activity was assayed by cup-plate or paper-disc method with *Bacillus subtilis* PCI 219 as a test organism and using the purest sample of sulfomycin I as a standard material.

As a starting material, 120-hour culture was used which was obtained by submerged-cultivation of the strain MCRL-0368 at 27°C in a liquid medium composed of glucose 4 %, sucrose 2 % and cotton seed meal 3 % (pH 7.0). Since the antibacterial activity was observed not only in the culture liquid but also in the mycelium, the whole broth (pH 7; 80 liters; 100 mcg/ml) was agitated with talc (1.6 kg) and Celite 545 (1.6 kg) and filtered with the aid of additional Celite 545 (5.0 kg). The cake collected was extracted twice with each 20 liters of acetone. The extract was concentrated *in vacuo* and the remaining aqueous solution (7 liters) was extracted twice with each 5 liters of ethyl acetate at pH 7.0. The extract (8.1 liters) was concentrated under reduced pressure to about 1 liter. The concentrate, after

Fig. 1. Thin-layer chromatographic behavior of sulfomycin I, II and III.
Kieselgel GF_{254}, $CHCl_3$-MeOH (10 : 1)
Detection by UV quenching
A: crude powder of sulfomycin complex.
B: sulfomycin I.
C: sulfomycin II.
D: sulfomycin III.

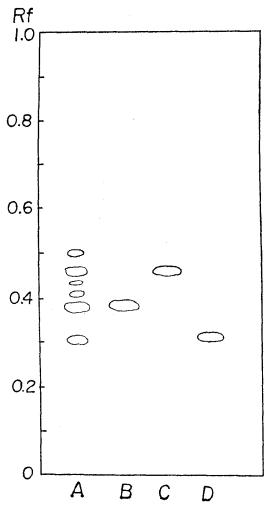


Table 1. Silicagel chromatography of sulfomycin mixture*

Fraction No. of eluates	Rf value of active principle(s) in the eluate**	Material recovered (mg)	MIC*** (mcg/ml)
83~119	0.49	9.5	25
120~145	0.46 (sulfomycin II)	220	0.2
146~184	0.46, 0.44, 0.42	205	3.1
185~189	0.42	16	1.0
198~230	0.38 (sulfomycin I)	1,800	0.1
305~350	0.30 (sulfomycin III)	30	6.25

* Silicagel (Kieselgel G): 400 g. Column size : 60×495 mm.
Temp. : 15°C.
Sulfomycin mixture used : 8,000 mg.
Volume of each fraction collected : 20 ml.
** Rf value on thin-layer chromatogram with Kieselgel GF_{254} (Solvent system : $CHCl_3$ - MeOH (10 : 1)).
*** Minimum inhibitory concentration against *B. subtilis* PCI 219 (Serial dilution method).

decolorization with aluminum oxide, was further evaporated *in vacuo* to give a syrup (30 ml), which solidified by treatment with 200 ml of ether. The solid material was collected and dried. Thus, the crude powder (12 g, 520 mcg/mg) of sulfomycin complex was obtained with over all recovery yield of about 78 %. Thin-layer chromatography with Kieselgel GF_{254} (solvent system : $CHCl_3$-MeOH (10 : 1), detection by UV lamp and bioautography) indicated that the crude powder was consisted of several biologically active principles showing Rf values of 0.30, 0.38, 0.42, 0.44, 0.46 and 0.49 (Fig. 1). The components showing Rf values at 0.38, 0.46 and 0.30 were the major components and designated sulfomycin I, II and III respectively.

Separation of each component was achieved by column chromatography with Kieselgel G (Merck) previously moistened with water and dried in air overnight. After washing the column with chloroform, development was made with a mixture of methanol and chloroform (3 : 100, v/v). Fractions of the eluates were monitored by thin-layer chromatography and bioautography. Active component(s) were recovered from the eluate by concentration followed by solidification of the resulting syrup with ethyl ether. Thus, starting from 8 g of crude powder, 1,800 mg of sulfomycin I, 220 mg of sulfomycin II and 30 mg of sulfomycin III were recovered as shown in Table 1. Attempts to crystallize the sulfomycins failed, so that further purification of the material was carried out by reprecipitation with acetone and ether.

Physicochemical Properties

Some of the physicochemical properties of sulfomycin I, II and III are summarized in Table 2. The ultraviolet and infrared absorption spectra are shown in Figs. 2 and 3, and NMR spectrum of I in Fig. 4. Based upon analytical data, empirical formulae $C_{55\sim57}H_{56\sim64}N_{15\sim17}O_{20\sim22}S_2$, $C_{45\sim47}H_{45\sim49}N_{12\sim14}O_{15\sim17}S_2$, $C_{50\sim52}H_{50\sim54}N_{13\sim15}O_{16\sim18}S_2$ are proposed

Tabel 2. Physicochemical properties of sulfomycin I, II and III

		Sulfomycin I	Sulfomycin II	Sulfomycin III
Appearance		colorless amorphous powder	colorless amorphous powder	colorless amorphous powder
Nature* (pka')		neutral or weakly acidic (11.10)	neutral or weakly acidic (11.30)	neutral or weakly acidic (10.90)
m. p. (decomp.)		no decomp. over 280°C	no definite m. p., decomp. at about 190°C	no definite m. p., decomp. at about 183°C
Elementary analysis (%)		C 49.95, 49.81 H 4.50, 4.49 N 16.86, 16.64 S 4.80, 4.58	C 50.68, 50.55 H 4.25, 4.17 N 16.14, 16.99 S 5.81, 5.31	C 50.42, 50.23 H 4.29, 4.32 N 16.71, 16.14 S 5.14, 5.46
M.W. (osmometry in chloroform)		1218, 1226	1138, 1139	1233
Optical rotation		$[\alpha]_D^{20}$ −16.0° (c 2, MeOH)	$[\alpha]_D^{27}$ −11.8° (c 1.19, MeOH)	$[\alpha]_D^{27}$ +3.2° (c 2.18, MeOH)
Ultraviolet absorption λ_{max} ($E_{1cm}^{1\%}$) (Fig. 2)	A**	252 mμ (712) and 325 mμ (sh, 110)	252 mμ (630) and 323 mμ (sh, 100)	252 mμ (672) and 324 mμ (sh, 94)
	B**	255 mμ (629) and 330 mμ (sh, 110)	256 mμ (549) and 328 mμ (sh, 103)	253 mμ (571) and 328 mμ (sh, 98)

* On paper electrophoresis (10 volt/cm, 2.5 hours in 1/15 M phosphate buffer), these three components did not move from the dotted point both at pH 5.0 and 8.0. However, on titration in 50 % EtOH, sulfomycins showed weakly acidic nature.

** A : Absorption in neutral or acidic methanol. B : Absorption in alkaline methanol.

for sulfomycin I, II and III respectively. Sulfomycins are readily soluble in acetone, dioxane, dimethylformamide, dimethylsulfoxide and acetic acid, soluble in lower alcohols, esters, chloroform and in dilute sodium hydroxide solution with decomposition, hardly soluble in water, and insoluble in aliphatic and aromatic hydrocarbons and ethers. Sulfomycins are stable in neutral or weakly acidic solution, but not in strongly acidic or alkaline solution.

Sulfomycins are positive to EHRLICH reaction and decolorize potassium permanganate solution. They give initially pale brown, later changing to reddish brown coloration with conc. sulfuric acid, while they are negative to ninhydrin, biuret (brown), ferric chloride, SAKAGUCHI, FEHLING, BENEDICT, TOLLENS, 2, 4-dinitrophenylhydrazine and concentrated hydrochloric acid reactions. Rf values are illustrated in Table 3. Summarized paper chromatogram of sulfomycin I is shown in Fig. 5, those of sulfomycin II and III resembling that of I.

Fig. 2. Ultraviolet absorption spectrum of sulfomycin I, II and III.

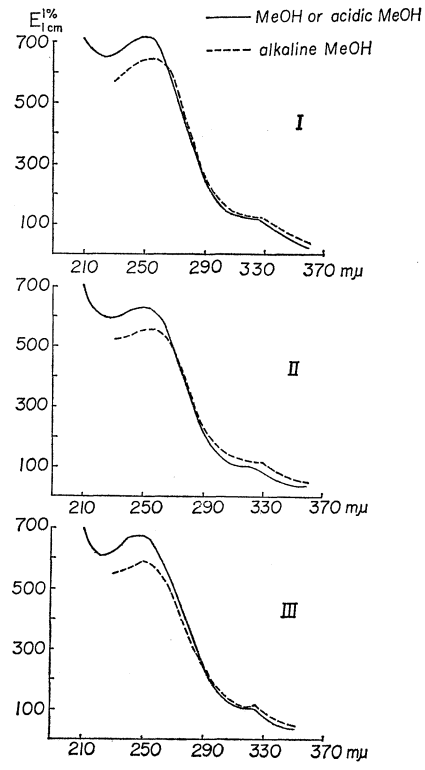
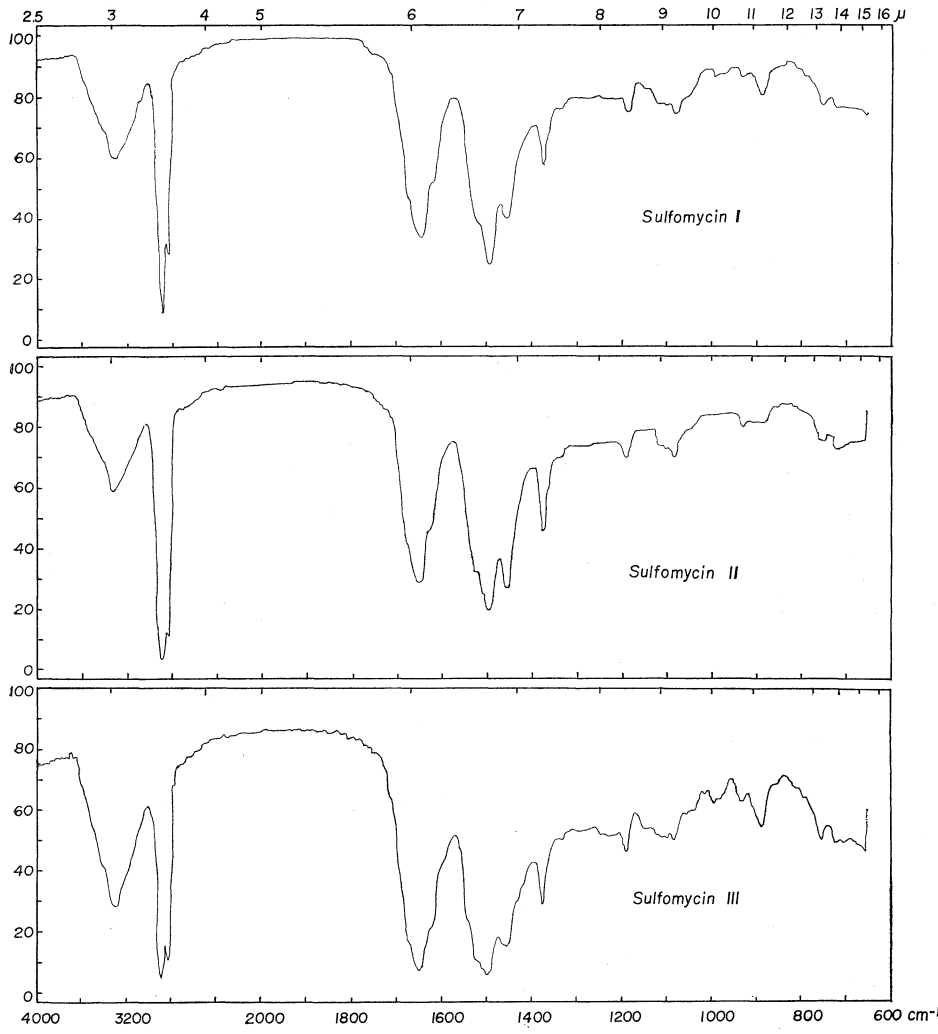


Fig. 3. Infrared spectra of sulfomycin I, II and III (Nujol)



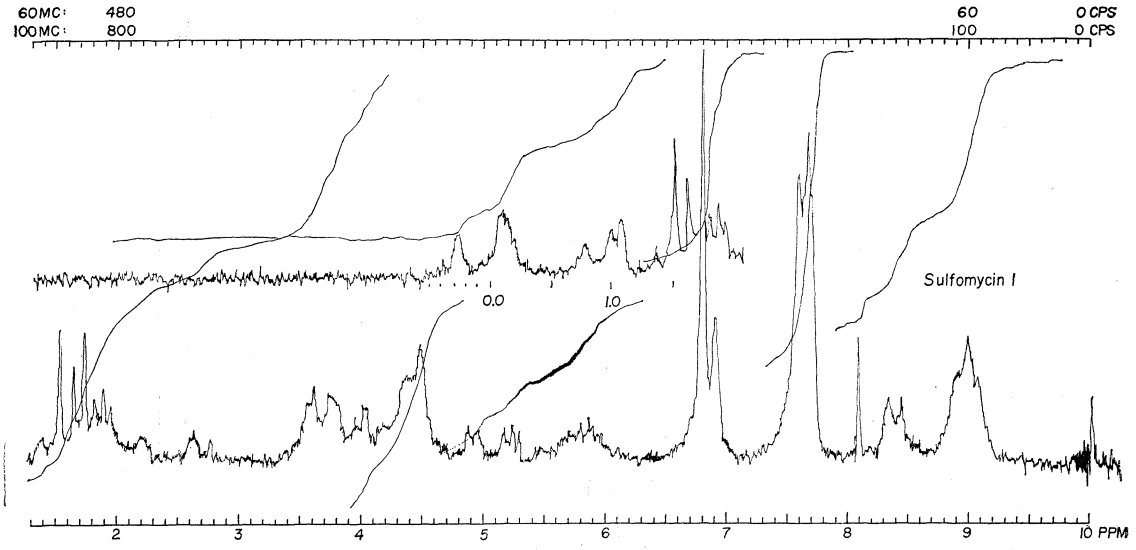
Automatic aminoacid analysis of acid hydrolysate (6 N HCl, 100°C for 12 hours) of sulfomycin I and II indicated that both antibiotics gave, as main degradation products, threonine and two unidentified ninhydrin-positive substance, one being neutral in nature and giving yellow color with ninhydrin reagent, while the other being basic and coloring violet with the reagent. In addition, the presence of minor amounts of alanine and glycine was also suggested.

Table 3. Rf values of sulfomycins on paper and thin-layer chromatographies (Bioautography with *B. subtilis*)

Solvent system		Sulfomycin		
		I	II	III
P. C.*	AcOEt saturated with H_2O	0.59	0.80	0.35
T.L.C.**	*n*-BuOH saturated with H_2O	0.70	0.80	0.63
	$CHCl_3$ - MeOH (10 : 1)	0.38	0.46	0.30
	AcOEt saturated with H_2O	0.13	0.26	0.05
	AcOEt - *n*-BuOH (1 : 1) saturated with H_2O	0.87	0.93	0.84

* Toyo Roshi No. 51 A : Ascending for 5 hours.
** Kieselgel GF_{254}.

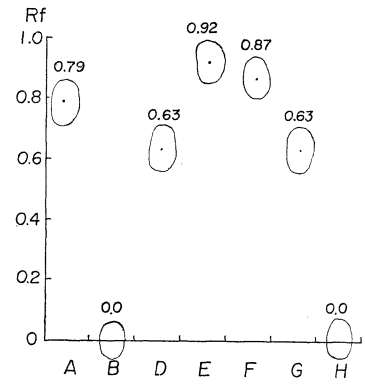
Fig. 4. NMR spectrum of sulfomycin I in d_6-DMSO with TMS (int.) (JEOL C-60 : 60 MC)



Column chromatography of the acid hydrolysate of sulfomycin I with cellulose powder (solvent systems : *n*-butanol – pyridine – water – acetic acid, 6 : 4 : 3 : 1) gave three discrete ninhydrin-positive degradation products, Rf values being 0.49, 0.30 and 0.21 (thin-layer chromatography on cellulose powder plate with solvent system as above). Substance with Rf 0.30 was identified as threonine and the others have not been identified yet. In addition, one unidentified ninhydrin-negative sulfur-containing substance was also isolated from the acid hydrolysate of sulfomycin I. Characterization of these unidentified substances is under study.

Fig. 5. Summarized paperchromatogram of sulfomycins (Bioautography with *B. subtilis*)

Solvent :
A : wet *n*-BuOH
B : 20 % ammonium chloride aq. solution
D : 50 % aq. acetone
E : *n*-BuOH(40 ml) - MeOH(10 ml) - H_2O(20 ml)+methylorange(1.5 g)
F : *n*-BuOH(40 ml) - MeOH(10 ml) - H_2O (20 ml)
G : benzene - MeOH (4 : 1)
H : H_2O



Biological Properties

Antimicrobial activities of sulfomycins are listed in Table 4. As evident from the table, they are strongly active against Gram-positive cocci and bacilli, mycoplasma and anaerobic bacteria, and weakly active against *Neisseria*, *Bordetella* and mycobacteria. But, they are inactive against *Escherichia*, *Pseudomonas*, fungi and yeast. They are active as well against strains resistant to penicillin, streptomycin, tetracycline and chloramphenicol and also against staphylococci freshly isolated from the clinical material at concentration of 0.19~0.39 mcg/ml. However, they showed cross resistance with bryamycin (thiostrepton).

Mice tolerated to the intraperitoneal administration of 400 mg/kg of sulfomycin I and III or 100 mg/kg of sulfomycin II. Survival effects were observed in mice infected

intraperitoneally with the mucin-added diffuse type of *Staphylococcus aureus* Smith, minimum effective dose of sulfomycins given intraperitoneally being 1.25 mg/kg for sulfomycin I and II, and 1.25~2.5 mg/kg for sulfomycin III.

Discussion

On the basis of biological properties, sulfomycin seemed to be related to the previously reported sulfur-containing peptidic antibiotics, bryamycin[1] (thiostrepton[2]), siomycin[3], pepthiomycin A and B[4] and substance A 59[5]. However, the above mentioned physicochemical properties of sulfomycins, especially amino-acid constitution and elementary analysis data, readily differentiate sulfomycins from these antibiotics and also from any of other known sulfur-containing antibiotics. Thus, the sulfomycins have been concluded to be a series of new sulfur-containing peptidic antibiotics.

Table 4. Antimicrobial activity of sulfomycins

Test Organisms *	MIC (mcg/ml) of sulfomycin		
	I	II	III
Staphylococcus aureus FDA 209P	0.19	0.19	0.78
// // Terashima	0.19	0.19	0.78
// // Smith	0.19	0.19	0.78
// // R 1	0.19	0.19	0.78
// // R 2	0.19	—	—
// // R 3	0.09	—	—
// // R 4	>25	>25	>25
Streptococcus hemolyticus	0.04	0.09	0.19
Diplococcus pneumoniae	0.04	0.09	0.39
Bacillus subtilis PCI 219	0.09	0.09	0.39
Corynebacterium diphtheriae Park-Williams No. 8	0.02	0.09	—
Bordetella pertussis Tohama	6.25 (48 hr)	6.25 (48 hr)	—
Neisseria meningitidis 13077	6.25	6.25	—
// // 13090	0.78	0.78	—
Clostridium tetani	0.39	6.25	—
Clostridium welchii	3.0	12.5	—
Mycobacterium tuberculosis $H_{37}Rv$	25.0 (3 weeks)	25.0 (3 weeks)	—
Escherichia coli NIHJ	>25	>25	>25
Pseudomonas aeruginosa	>25	>25	>25
Aspergillus niger	>25	>25	>25
Penicillium notatum	>25	>25	>25
Candida albicans	>25	>25	>25
Saccharomyces cerevisiae	>25	>25	>25
Mycoplasma galisepticum	0.06 (48 hr)	—	—

* R 1 : penicillin, streptomycin, tetracycline and chloramphenicol-resistant strain
R 2 : amphomycin-resistant strain
R 3 : telomycin-resistant strain
R 4 : bryamycin-resistant strain

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