ISOLATION OF AN ANTIBIOTIC S-583-B, RELATED TO RHODOMYCIN A AND B

JUN'ICHI SHOJI, SHUICHI KOZUKI, HARUO NISHIMURA, MIKAO MAYAMA, KIYOSHI MOTOKAWA, YŪ TANAKA and HIDEO OTSUKA

Shionogi Research Laboratory, Shionogi & Co., Ltd., Fukushima-ku, Osaka, Japan

(Received for publication September 11, 1968)

From a streptomyces strain identified as *Streptomyces purpurascens*, an anthracycline antibiotic named S-583-B was isolated together with rhodomycin A and B. The chromophore of the antibiotic S-583-B was proved to be identical or closely similar to β-rhodomycinone, but the sugar moiety was found to consist of several sugar components including an aminosugar found to be rhodosamine.

In the course of our screening for new antibiotics, a streptomyces strain S-583 isolated from a soil sample collected at Kyoto City was found to produce several red pigments which exhibited antibiotic activities. The strain was proved to be identical with *Streptomyces purpurascens* by comparative experiments using the type culture.

The red antibiotics of basic nature produced by the fermentation of this strain were extracted with ethyl acetate and then transferred into dilute hydrochloric acid. The antibiotic mixture present as the hydrochloride salts was then distributed into two fractions according to their solubility properties in chloroform. From the chloroform-soluble fraction, a red basic antibiotic was isolated and named S-583-B. The chloroform-insoluble fraction contained multiple components and was tentatively named S-583-A complex, from which two main components, S-583-A-II and III, were isolated by column chromatography on metal-free silica gel, and were found identical to rhodomycin B and A[1,2,3], respectively.

Thin-layer chromatograms on a metal-free silica gel[4] plate, where S-583-B was distinguishable from S-583-A-II and III, are illustrated in Fig. 1.

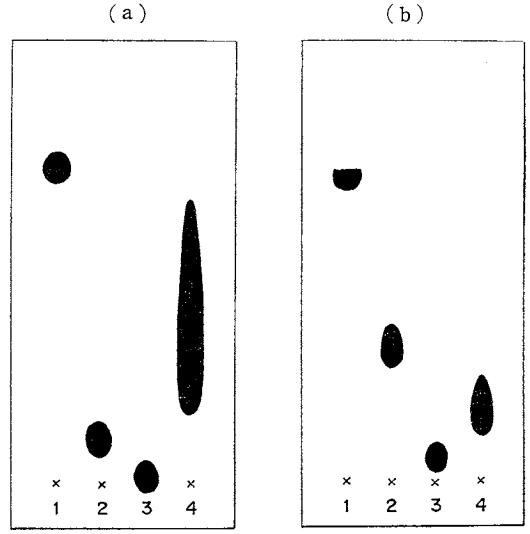
The antimicrobial spetrum of S-583-B hydrochloride (Table 1) indicated this antibiotic was mainly active against gram positive bacteria. Acute toxicity studies in mice gave an LD_{50} of 1~2 mg/kg intraperitoneally.

The antibiotic S-583-B hydrochloride was obtained as a red amorphous powder, which melted with decomposition at 184~190°C, and was optically active, $[\alpha]_D^{24}$ +122° ±50° (*c* 0.0106, chloroform). Elemental analysis and molecular weight determination with a sample equilibrated to atmospheric environment gave the following results: C 54.75, H 6.96, N 2.06, Cl 5.51, H_2O 6.28%; MW, 1298 (osmometry in chloroform), and 629 (osmometry in 95% methanol). A molecular weight 1210 was calculated from an absorption at 496 mμ. The ultraviolet and infrared absorption spectra are shown in Figs. 2 and 3 respectively.

Table 1. Antimicrobial spectrum of 583-B hydrochloride

Test organism	M. I. C. (mcg/ml)
Shigella dysenteriae	>50.0
Salmonella typhosa	>50.0
Escherichia coli, UMEZAWA	>50.0
Pseudomonas aeruginosa	>50.0
Klebsiella pneumoniae	>50.0
Bacillus subtilis, PCI-219	0.5
Bacillus anthracis	0.5
Staphylococcus aureus, FDA 209P	0.5
Sarcina lutea, PCI-1001	1.0
Diplococcus pneumoniae, Type I	0.5
Streptococcus hemolyticus, Denken	1.0
Corynebacterium diphtheriae, Tront	1.0

Fig. 1. Thin-layer chromatograms on a metal-free silica gel plate.
(a) Developed with chloroform-methanol (85 : 15)
(b) Developed with benzene-ethyl formate-formic acid (3 : 2 : 2)
1: Chromophores of S-583-A-II and III, and S-583-B
2: S-583-A-II hydrochloride
3: S-583-A-III hydrochloride
4: S-583-B hydrochloride



S-583-B hydrochloride is soluble in water, methanol, ethanol, chloroform and dimethylformamide, and slightly soluble in acetone, but not soluble in ethyl acetate, ether, benzene and *n*-hexane.

Basic nature was indicated by paper electrophoresis carried out in N acetic acid. This antibiotic is red in acid solutions and bluish purple in alkaline solutions, and gives reddish purple with a methanol solution of magnesium acetate.

When S-583-B was hydrolyzed with dilute hydrochloric acid, a chromophore, whose absorption maxima at ultraviolet and visible regions were practically identical with the parent antibiotic, was isolated as red fine crystals, m. p. 220~226°C (dec.) sintering at 190°C. Elemental analysis and mass spectroscopy indicated a molecular formula $C_{20}H_{18}O_8$ for this chromophore. The infrared absorption spectrum (Fig. 4) and n.m.r. spectrum (Fig. 5) were assignable to the proposed structure[6] of β-rhodomycinone[5,6]. Furthermore, the identity of this chromophore and the chromophore similarly derived from S-583-A-II and III (rhodomycin B and A) was confirmed by thin-layer chromatographic comparison as well as their physico-chemical properties.

From these, the chromophore of S-583-B was deduced to be probably β-rhodomycinone, although its stereochemistry is as yet somewhat obscure in the present data.

Fig. 2. Ultraviolet absorption spectrum of S-583-B hydrochloride measured in methanol.

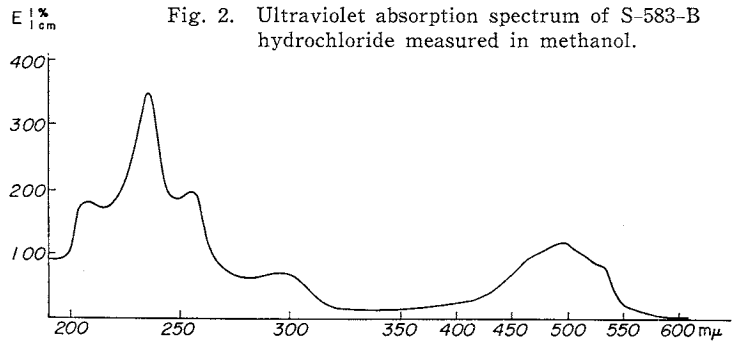


Fig. 3. Infrared absorption spectrum of S-583-B hydrochloride (Chloroform solution)

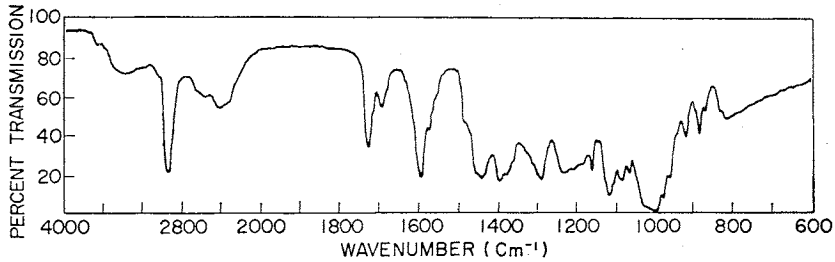


Fig. 4. Infrared absorption spectrum of the chromophore of S-583-B (KBr tablet)

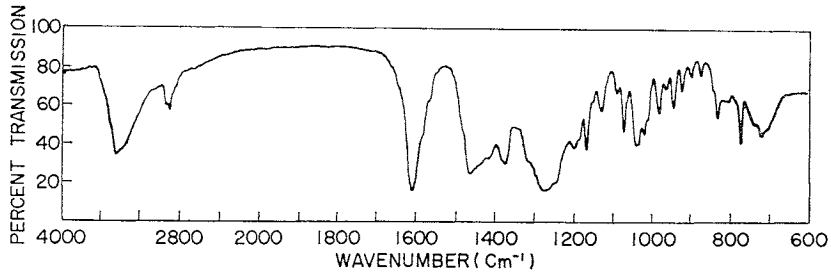
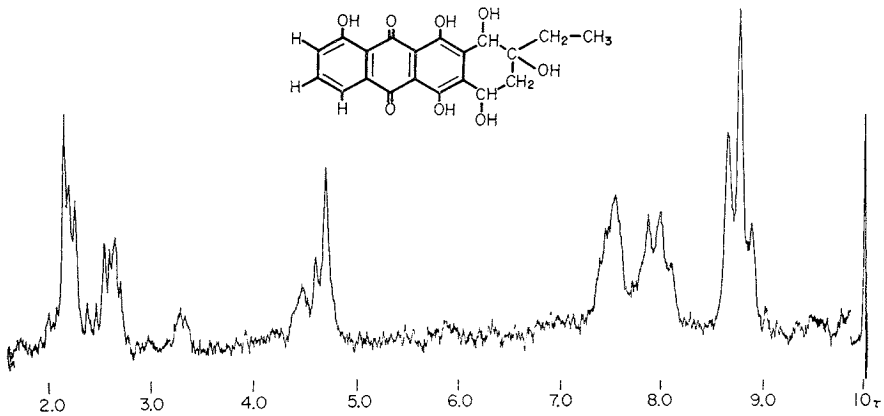


Fig. 5. N.m.r. spectrum of the chromphore of S-583-B.
This spectrum was taken with a Varian A-60 spectrometer on solution in trifluoroacetic acid containing tetramethylsilane as an internal reference at normal probe temperature.



The remaining part of the hydrolysate of S-583-B, which was reasonably considered to be the sugar moieties of this anthracycline, was examined by paper electrophoresis and by gas-liquid chromatography of their trimethylsilyl derivatives (Fig. 6). These results suggested that several sugar components were contained including neutral and basic sugars, one of which could be inferred to be rhodosamine[3,7].

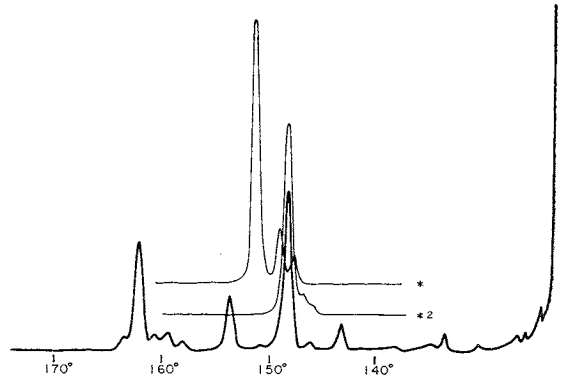
2-Deoxy-L-fucose[7] has been reported to be a sugar component of anthracycline antibiotics such as cinerubin A and B, and γ-rhodomycin III and IV. The absence of this sugar in the hydrolysate of S-583-B was proved by comparative gas-liquid chromatographic analysis using a synthesized preparation of 2-deoxy-D-fucose whose retention time must be the same one as that of its L-enanthiomer.

All the above results indicated that the antibiotic S-583-B is closely similar to rhodomycin A and B, but different in the constituents of its sugar moiety. Many anthracycline antibiotics from actinomycete have been reported up to this time. However, only rhodomycin A and B have been known to have β-rhodomycinone as their chromophore. It is characteristic of the strain S-583, identified as *Streptomyces purpurascens*, that it produces antibiotic S-583-B and rhodomycin A and B, but does not produce iso-rhodomycin A and B[7].

Fig. 6. Gas-liquid chromatography of the sugar moiety of S-583-B.

* 2-Deoxy-D-fucose

** An aminosugar of S-583-A-II and III.



Experimental

Taxonomic studies on the S-583-B producing strain:

Morphological, cultural and physiological characteristics of the strain were studied and the results indicated it to be the same species as *Streptomyces purpurascens* NRRL B1454 by direct comparison.

Fermentation:

The streptomyces strain S-583 was shake-cultured for 48 hours at 27°C with a medium consisting of 2.0 % glucose, 0.5 % meat extract, 0.5 % peptone, 0.5 % sodium chloride and 0.35 % calcium carbonate, pH 7.0. A 450-ml portion of the culture was then transferred to a 30-liter jar fermentor containing 15 liters of a medium consisting of 1.0 % starch, 0.5 % glycerine, 1.0 % soy bean meal, 0.5 % corn steep liquor, 0.3 % sodium chloride and 0.3 % calcium carbonate, pH 7.0. Fermentation was carried out for 5 days at 28°C under agitation of 250 r.p.m. and aeration of 20 liters per minute.

Preparation of crude materials:

About 50 liters of the cultured broth obtained as above was adjusted to pH 3.0 by dilute hydrochloric acid and filtered. The mycelial cake was twice extracted with methanol (3 liters) at pH 3.0. The methanol solution was evaporated to a nearly aqueous solution, and the red pigments contained was transferred to ethyl acetate by adjusting to pH 7.0~7.5. The filtrate was also extracted with ethyl acetate (10 liters) at pH 7.0~7.5. The both ethyl acetate solutions of red pigments were then combined and concentrated to a small volume. After drying with anhydrous sodium sulfate, the solution was further concentrated to an oily material, from which a red precipitate (3.65 g) was produced by the addition of petroleum ether.

The above precipitate was dissolved into ethyl acetate, and then the solution was shaken with water acidified to pH 2.0 by hydrochloric acid. Almost all the pigments were transferred into the water, and the water solution was then repeatedly extracted with chloroform maintaining an acid pH. By this procedure, the red pigments were distributed to the water and also to the chloroform solutions.

The above chloroform solution was dried and concentrated. A crude material (501 mg) of S-583-B hydrochloride was precipitated as a red amorphous powder by the addition of petroleum ether.

The remaining pigments in the acid water solution was extracted with chloroform by adjusting to pH 7.0. The chloroform extract was dried and concentrated, and a crude

material (476 mg) of S-583-A-complex was obtained as a red amorphous powder by precipitation with petroleum ether.

Purification of S-583-B:

The crude material of S-583-B hydrochloride (500 mg) obtained by the above procedure was dissolved in dilute hydrochloric acid. Extraction with chloroform was repeated, the chloroform solution was dried and concentrated, and a red precipitate was produced by the addition of ethyl ether. Dissolution into a small volume of chloroform and precipitation by ethyl ether were repeated. Finally, a purified preparation of S-583-B hydrochloride was obtained as a red amorphous powder (282 mg), m. p. 184~190° (dec.), $[\alpha]_D^{24}$ +122°±50° (*c* 0.0106, chloroform).

Anal. Found: C 54.75, H 6.96, N 2.06, Cl 5.51, H_2O 6.28 %, MW, 1,298 (osmometry in chloroform), 629 (osmometry in 95 % methanol).

Purification of S-583-A-II and III, and identification with rhodomycin B and A:

The crude preparation of S-583-A complex was chromatographed on a metal-free silica gel column with chloroform-methanol (7 : 3) acidified by hydrogen chloride. Two main components, tentatively named S-583-A-II and S-583-A-III, were isolated as their hydrochlorides. Their physico-chemial properties were as follows:

S-583-A-II hydrochloride; red amorphous powder, m. p. 185~190°C (dec.), $[\alpha]_D^{23}$ +273° ±62° (*c* 0.011, methanol). λ_{max}^{MeOH}: 235, 254, 294, 495, 528 (shoulder), and 562 mμ (shoulder).

Anal. Found: C 57.05, H 5.23, N 2.37, Cl 7.22 %. Molecular ion peak: 543.

Calcd. for $C_{28}H_{33}O_{10}N \cdot HCl$: C 57.93, H 5.86, N 2.41, Cl 6.11 %, $C_{28}H_{33}O_{10}N$: MW 543.55.

S-583-A-III dihydrochloride; red amorphous powder, m. p. 188~192°C (dec.). $[\alpha]_D^{23}$ +418°±74° (*c* 0.011, methanol). λ_{max}^{MeOH}: 236, 255, 295, 497, 530 (shoulder), and 564 mμ (shoulder).

Anal. Found: C 55.26, H 5.90, N 3.44, Cl 9.10.

Calcd. for $C_{36}H_{48}O_{12}N_2 \cdot 2HCl$: C 55.87, H 6.47, N 3.62, Cl 9.18 %.

These properties of S-583-A-II and III were practically similar to those described in the earlier reports[1,2] of rhodomycin B and A, and, especially, the elemental analysis and the molecular weight supported by mass spectrometry were consistent with the proposed structures[3].

Coversion of S-583-A-III to S-583-A-II and finally to a chromophore, which was inferred to be β-rhodomycinone, was observed by mild acid hydrolysis. Furthermore, authentic samples of rhodomycin A and B (kindly supplied by Prof. H. BROCKMANN) moved similarly to S-583-A-III and II positions, respectively, on thin-layer chromatography (Fig. 1). From these observations, it was deduced that S-583-II and III were identical with rhodomycin B and A, respectively.

Examinations on the acid hydrolysate of S-583-B:

(**a**) On the chromophore. S-583-B hydrochloride (100 mg) was hydrolyzed with 0.2 N HCl (5 ml) at 80~85°C for one hour. A red precipitate (32 mg) produced was collected and crystallized from methanol-chloroform to yield red fine crystals (17 mg), m. p. 220~226°C (dec.) sintering at 190°C.

Anal. Found: C 61.67, 62.05, H 4.72, 4.49, MW 386 (mass spectroscopy).

Calcd. for $C_{20}H_{18}O_8$: C 62.17, H 4.70 %.

λ_{max}^{MeOH} mμ (ε): 234.5 (3.96×10^4), 254 (2.28×10^4), 293 (7.2×10^3), 495 (1.42×10^4), 529 (9.8×10^3). The above properties and thin-layer chromatographic mobilities as well as infrared absorption spectrum (Fig. 4) and n.m.r. spectrum (Fig. 5) were all identical with those of a chromophore prepared from S-583-A-II and III in a similar way.

(**b**) On the sugar moiety. The supernatant portion of the hydrolysate of S-583-B mentioned above was further hydrolyzed with 2 N hydrochloric acid for one hour at 100°C.

The resulting hydrolysate was examined by paper electrophoresis carried out at 300 volt for 2 hours in N acetic acid. One spot each of neutral and basic substances was detected by potassium permanganate solution.

Further, the hydrolysate was de-acidified with IR-4B (OH^-), lyophilized and then trimethylsilylated with 25 % bis-(trimethylsilyl)-acetamide in pyridine by heating at 75°C for 20 minutes. The trimethylsilyl derivatives were then subjected to gas-liquid chromatography carried out by a Perkin-Elmer Model 881 on a 6 ft glass column packed with 2.5 % SE-30 coated Chromosorb P, programed from 120°C to 250°C at 4°C per minute. Three or more peaks were observed on the chromatograph chart (Fig. 6). One peak coincided with that of an aminosugar of S-583-A-II and III (rhodomycin B and A), which was, therefore, considered to be rhodosamine. A synthesized sample of 2-deoxy-D-fucose was also run as reference, but the peak did not correspond to any of the peaks of the S-583-B hydrolysate.

Acknowledgement

The authors express their sincere thanks to Prof. H. BROCKMANN, Göttingen Universität for his kind gift of rhodomycin A and B. The authors also are indebted to the following members of our laboratory: Dr. Y. NOZAKI for the sample of 2-deoxy-D-fucose and Mr. N. SHIMAOKA for the experiment on toxicity.

References

1) BROCKMANN, H. & I. BORCHERS: Rhodomycin, II. Chem. Ber. 86 : 261~269, 1953.
2) BROCKMANN, H. & P. PATT: Iso-rhodomycin A, ein neues Antibioticum aus *Streptomyces purpurascens*, Rhodomycine, III. Chem. Ber. 88 : 1455~1468, 1955.
3) BROCKMANN, H. & E. SPOHLER: Zur Konstitution des Rhodomycins A. Naturwiss. 48 : 716~717, 1961.
4) SHŌJI, J.; Y. KIMURA & K. KATAGIRI: Julymycin B-II, a new antibiotic. Isolation and characterization. J. Antibiotics, Ser. A 17 : 156~160, 1964.
5) BROCKMANN, H. & B. FRANCK: Rhodomycinone und Isorhodomycinone, Rhodomycine, IV. (Antibiotica aus Actinomyceten, XXXIII) Chem. Ber. 88 : 1792~1818, 1955.
6) BROCKMANN, H. & J. NIEMEYER: Zur Konstitution des β- und γ-Rhodomycinons. Naturwiss. 48 : 570~571, 1961.
7) BROCKMANN, H.: Anthracyclinone und Anthracycline (Rhodomycinone, Pyrromycinone und ihre Glykoside). Fortsch. Chem. Org. Naturstoff. 21 : 121~179, 1963.